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MODULATION OF SODIUM CHANNELS IN DORSAL ROOT GANGLIA

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## **Modulation of Sodium Channels in Dorsal Root Ganglia**

**by Sulayman Dib-Hajj and Stephen G. Waxman**

### **FIELD OF THE INVENTION**

The present invention relates to a novel tetrodotoxin resistant sodium  
5 channel and related nucleotides, as well as screening assays for identifying  
agents useful in treating acute or chronic pain or other hyperexcitability states.  
This application is related to U.S. Provisional Application 60/072,990, filed  
January 29, 1998, U.S. Provisional Application 60/109,402 entitled  
“Modulation of Sodium Channels in Dorsal Root Ganglia”, filed November 20,  
10 1998 and to U.S. Provision Application 60/109,666, entitled “Differential Role  
of GDNF and NGF in the Maintenance of Two TTX-Resistant Sodium  
Channels in Adult DRG Neurons,” filed on November 20, 1998, all of which  
are herein incorporated by reference.

### **BACKGROUND**

#### **15 A. Sodium Channels**

Voltage-gated sodium channels are a class of specialized protein  
molecules that act as molecular batteries permitting excitable cells (neurons and  
muscle fibers) to produce and propagate electrical impulses. Voltage-gated Na<sup>+</sup>  
channels from rat brain are composed of three subunits, the pore-forming  $\alpha$   
20 subunit (260 KDa) and two auxillary subunits,  $\beta$ 1 (36 KDa) and  $\beta$ 2 (33 KDa)  
that may modulate the properties of the  $\alpha$ -subunit; the  $\alpha$  subunit is sufficient to  
form a functional channel that generates a Na current flow across the membrane  
[references 1,2 as cited below]. Nine distinct  $\alpha$  subunits have been identified in  
vertebrates and are encoded by members of an expanding gene family [3 and  
25 references therein, 4-6] and respective orthologues of a number of them have  
been cloned from various mammalian species including humans. Specific  $\alpha$   
subunits are expressed in a tissue- and developmentally-specific manner [7,8].

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Aberrant expression patterns or mutations of voltage-gated sodium channel  $\alpha$ -subunits underlie a number of human and animal disorders [9-13].

Voltage-gated sodium channel  $\alpha$ -subunits consist of four domains (D1-4) of varying internal homology but of similar predicted structure, 5 connected by three intracellular loops (L1-3). The four domains fold to form a channel that opens to both the cytoplasm and the extracellular space via a pore. The pore opens and closes depending upon the physiological state of the cell membrane.

Each domain consists of six transmembrane segments (S1-6) that allow 10 the protein to weave through the membrane with intra- and extracellular linkers. The linkers of S5-S6 segments of the four domains contain sequences that line the pore of the channel, and a highly conserved subset of amino acids that acts as a filter to selectively allow sodium ions to traverse the channel pore into the cytoplasm, thus generating an electric current. The amphipathic S4 segment, in 15 each of the four domains, rich in basic residues repeated every third amino acid, acts as a voltage sensor and undergoes a conformational change as a result of the change in the voltage difference across the cell membrane. This in turn triggers the conformational change of the protein to open its pore to the extracellular  $\text{Na}^+$  ion gradient.

20 In most of the known voltage-gated sodium channel  $\alpha$ -subunits, the channels close and change into an inoperable state quickly (inactivate), within a few milliseconds, after opening of the pore (activation); SNS-type channels, on the other hand, inactivate slowly and require a greater voltage change to activate. L3, the loop that links domains D3 and D4, contains a tripeptide 25 which acts as an intracellular plug that closes the pore after activation, thus inducing the channel to enter the inactive state. After inactivation, these channels further undergo conformational change to restore their resting state and become available for activation. This period is referred to as recovery from inactivation (repriming). Different channels reprime at different rates, and 30 repriming in SNS is relatively rapid.

Based on amino acid similarities, the voltage-gated sodium channel family has been further subdivided into two subfamilies [14]. Eight of the nine cloned channels belong to subfamily 1. They share many structural features, particularly in their S4 transmembrane segments. However, some of them have  
5 been shown to have distinct kinetic properties of inactivation and repriming. Only a single channel of subfamily 2, also referred to as atypical channels, has been identified in human, rat and mouse tissues. This subfamily is primarily characterized by reduced numbers of basic residues in its S4 segments, and thus is predicted to have different voltage-dependence compared to subfamily 1. The  
10 physiological function of subfamily 2 channels is currently unknown because its electrophysiological properties have not yet been elucidated.

The blocking of voltage-gated sodium channels by tetrodotoxin, a neurotoxin, has served to functionally classify these channels into sensitive (TTX-S) and resistant (TTX-R) phenotypes. Two mammalian TTX-R channels  
15 have so far been identified, one specific to the cardiac muscle and to very limited areas of the central nervous system (CNS) and the second, SNS, is restricted to peripheral neurons (PNS) of the dorsal root ganglia (DRG) and trigeminal ganglia. Specific amino acid residues that confer resistance or sensitivity to TTX have been localized to the ion selectivity filter of the channel  
20 pore. The SNS channel is also described in International Patent Application WO 97/01577.

#### **B. Role of Sodium Channels in Disease States**

Because different Na<sup>+</sup> channel  $\alpha$ -subunit isotypes exhibit different kinetics and voltage-dependence, the firing properties of excitable cells depend  
25 on the precise mixture of channel types that they express. Mutants of the cardiac and skeletal muscle  $\alpha$ -subunit have been shown to cause a number of muscle disorders. Some examples are as follows: A change of a single basic amino acid residue in the S4 of the skeletal muscle channel is sufficient to change the kinetic properties of this channel and induce a disease state in many

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patients. A tripeptide deletion in L3 of the cardiac channel, proximal to the inactivation gate, induces a cardiac disorder called Long QT syndrome. A single amino acid change in the S5-S6 linker of domain 1 of Scn8a, the region lining the pore of the channel, causes the mouse mutant "jolting". The total loss  
5 of this channel by a different mutation causes motor end plate "med" disease in mice. This mutation is characterized by loss of motor neuron stimulation of the innervated muscle.

### C. Sodium Channels and Pain

Axonal injury (injury to nerve fibers, also called axons) can produce  
10 chronic pain (termed neuropathic pain). A number of studies have demonstrated altered excitability of the neuronal cell body and dendrites after axonal injury [15-17], and there is evidence for a change in Na<sup>+</sup> channel density over the neuronal cell body and dendrites following axonal injury [18-20]. The expression of abnormal mixtures of different types of sodium channels in a  
15 neuronal cell can also lead to abnormal firing [13], and can contribute to hyperexcitability, paresthesia or pain.

Recent studies from our group on rat sensory DRG neurons have demonstrated a dramatic change in the expression profile of TTX-R and TTX-S currents and in a number of mRNA transcripts that could encode the channels  
20 responsible for these currents in DRG neurons following various insults [21-23]. We have, for example, shown an attenuation of the slowly inactivating, TTX-R current and simultaneous enhancement of the rapidly inactivating, TTX-S Na<sup>+</sup> currents in identified sensory cutaneous afferent neurons following axotomy [21]. We also have shown a loss of TTX-S, slowly repriming current and  
25 TTX-R current and a gain in TTX-S, rapidly repriming current in nociceptive (pain) neurons following axotomy [22], down-regulation of SNS transcripts and a simultaneous up-regulation of  $\alpha$ -III Transcripts [23]. Also associated with axotomy is a moderate elevation in the levels of  $\alpha$ I and  $\alpha$ II mRNAs [24]. These changes in the sodium channel profile appear to contribute to abnormal

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firing that underlies neuropathic pain that patients suffer following axonal injury.

Inflammation, which is also associated with pain (termed inflammatory pain), also causes alteration in the sodium current profile in nociceptive DRG neurons. Inflammatory modulators up-regulate TTX-R current in small C-type nociceptive DRG neurons in culture [25,26]. The rapid action of these modulators suggests that their action include posttranslational modification of existing TTX-R channels. We have now determined that inflammation also increases a TTX-R  $\text{Na}^+$  current and up-regulates SNS transcripts in C-type DRG neurons [58]. This data suggests that changes in the sodium current profile contribute to inflammation evoked-pain.

#### **D. Therapies for Chronic Pain:**

A variety of classes of drugs (anticonvulsants such as phenytoin and carbamazepine; anti-arrhythmics such as mexitine; local anesthetics such as lidocaine) act on  $\text{Na}^+$  channels. Since the various  $\text{Na}^+$  channels produce sodium currents with different properties, selective blockade or activation (or other modulation) of specific channel subtypes is expected to be of significant therapeutic value. Moreover, the selective expression of certain  $\alpha$ -subunit isoforms (PN1, SNS, NaN) in specific types of neurons provides a means for selectively altering their behavior.

Nociceptive neurons of the DRG are the major source of the PNS TTX-R  $\text{Na}^+$  current. Thus, the  $\text{Na}^+$  channels producing TTX-R currents provide a relatively specific target for the manipulation of pain-producing neurons. The molecular structure of one TTX-R channel in these DRG neurons, SNS, has been identified but, prior to our research, it has not been determined whether there are other TTX-R channels in these neurons. If such channels could be identified, they would be ideal candidates as target molecules that are preferentially expressed in nociceptive neurons, and whose modulation would attenuate pain transmission.



## SUMMARY OF THE INVENTION

The present invention includes an isolated nucleic acid which encodes a voltage gated  $\text{Na}^+$  channel that is preferentially expressed in dorsal root ganglia or trigeminal ganglia (the NaN channel). (In our preceding U.S. Provisional  
5 Application 60/072,990, this NaN channel was referred to by its previous name "NaX.") In a preferred embodiment, the isolated nucleic acid comprises the sequence shown in Fig. 1, Fig. 7A, Fig. 8A, allelic variants of said sequences or nucleic acids that hybridize to the foregoing sequences under stringent conditions.

10 In another embodiment, the invention includes an expression vector comprising an isolated nucleic acid which encodes the voltage gated  $\text{Na}^+$  channel that is preferentially expressed in dorsal root ganglia or trigeminal ganglia either alone or with appropriate regulatory and expression control elements. In a preferred embodiment, the expression vector comprises an  
15 isolated nucleic acid having the sequence shown in Fig. 1, Fig. 7A, Fig. 8A, allelic variants of said sequences or nucleic acids that hybridize to the foregoing sequences under stringent conditions.

The present invention further includes a host cell transformed with an expression vector comprising an isolated nucleic acid which encodes a voltage  
20 gated  $\text{Na}^+$  channel that is preferentially expressed in dorsal root ganglia or trigeminal ganglia with appropriate regulatory and expression control elements. In a preferred embodiment, the expression vector comprises an isolated nucleic acid having the sequence shown in Fig. 1, Fig. 7A, Fig. 8A, allelic variants of said sequences or nucleic acids that hybridize to the foregoing sequences under  
25 stringent conditions.

The present invention also includes an isolated voltage gated  $\text{Na}^+$  channel that is preferentially expressed in dorsal root ganglia or trigeminal ganglia. In a preferred embodiment, the channel has the amino acid sequence of Figs. 2, 7B or 8B or is encoded by a nucleic acid having the sequence shown in  
30 Figs. 1, 7A or 8A, allelic variants of said sequences or nucleic acids that

hybridize to the foregoing sequences under stringent conditions. Peptide fragments of the channel are also included.

Another aspect of the invention is a method to identify an agent that modulates the activity of the NaN channel, comprising the steps of bringing the agent into contact with a cell that expresses the Na<sup>+</sup> channel on its surface and measuring depolarization, or any resultant changes in the sodium current. The measuring step may be accomplished with voltage clamp measurements, by measuring depolarization, the level of intracellular sodium or by measuring sodium influx.

Another aspect of the invention is a method to identify an agent that modulates the transcription or translation of mRNA encoding the NaN channel. The method comprises the steps of bringing the agent into contact with a cell that expresses the Na<sup>+</sup> channel on its surface and measuring the resultant level of expression of the Na<sup>+</sup> channel.

The invention also includes a method to treat pain, paraesthesia and hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of modulating, such as by inhibiting or enhancing, Na<sup>+</sup> current flow through NaN channels in DRG or trigeminal neurons. The method may include administering an effective amount of an agent capable of modulating the transcription or translation of mRNA encoding the NaN channel.

Another aspect of the invention is an isolated nucleic acid that is antisense to the nucleic acids described above. In a preferred embodiment, the antisense nucleic acids are of sufficient length to modulate the expression of NaN channel mRNA in a cell containing the mRNA.

Another aspect of the invention is a scintigraphic method to image the loci of pain generation or provide a measure the level of pain associated with DRG or trigeminal neuron mediated hyperexcitability in an animal or human subject by administering labeled monoclonal antibodies or other labeled ligands specific for the NaN Na<sup>+</sup> channel.

Another aspect of the invention is a method to identify tissues, cells and cell types that express the NaN sodium channel. This method comprises the step of detecting NaN on the cell surface, or en route to the cell surface, or the presence of NaN encoding mRNA.

5       The present invention further includes a method of producing a transformed cell that expresses an exogenous NaN encoding nucleic acid, comprising the step of transforming the cell with an expression vector comprising an isolated nucleic acid having the sequence shown in Figs. 1, 7A or 8A, allelic variants of said sequences or nucleic acids that hybridize to the  
10 foregoing sequences under stringent conditions, together with appropriate regulatory and expression control elements. The invention also includes a method of producing recombinant NaN protein, comprising the step of culturing the transformed host under conditions in which the NaN sodium channel or protein is expressed, and recovering the NaN protein.

15       The invention also includes an isolated antibody specific for the *NaN* channel or polypeptide fragment thereof. The isolated antibody may be labeled.

Another aspect of the invention includes a therapeutic composition comprising an effective amount of an agent capable of decreasing rapidly repriming sodium current flow in axotomized, inflamed or otherwise injured  
20 DRG neurons or in normal DRG neurons that are being driven to fire at high frequency. The invention also includes a method to treat acute pain or acute or chronic neuropathic or inflammatory pain and hyperexcitability phenomena in an animal or a human patient by administering the therapeutic composition.

The present invention also includes a method to screen candidate  
25 compounds for use in treating pain and hyperexcitability phenomena by testing their ability to alter the expression or activity of an NaN channel mRNA or protein in axotomized, inflamed or otherwise injured DRG neurons.

## BRIEF DESCRIPTION OF THE DRAWING FIGURES

Fig. 1 shows the sequence of the rat *NaN* cDNA.

Fig. 2 shows the putative amino acid sequence of the rat *NaN* cDNA. Predicted transmembrane segments of domains I - IV are underlined. The amino acid serine "S" in DI-SS2, implicated in the TTX-R phenotype, is in bold face type.

- 5     Fig. 3 presents a schematic diagram of predicted secondary structure of the *NaN*  $\alpha$ -subunit.

Fig. 4 shows the results of RT-PCR analysis for  $\alpha$ -*NaN* in extracts of various tissues using *NaN*-specific primers. *NaN* is abundantly expressed in dorsal root and trigeminal ganglia. Low levels of *NaN* are detected in cerebral hemisphere  
10 and retina tissues. No detectable *NaN* signal is seen in cerebellum, optic nerve, spinal cord, sciatic nerve, superior cervical ganglia, skeletal muscle, cardiac muscle, adrenal gland, uterus, liver and kidney.

Fig. 5 shows the tissue distribution of  $\alpha$ -*NaN* by *in situ* hybridization. A. Trigeminal ganglion neurons show moderate-to-high hybridization signal. B.  
15 Dorsal root ganglion neurons show moderate-to-high hybridization signal in small neurons. Hybridization signal is attenuated in large neurons (arrow). C. Sense probe shows no signal in DRG neurons. D., E., and F. No hybridization signal is seen in spinal cord, cerebellum and liver. All tissues are from adult Sprague-Dawley rat. Scale bars = 50 micrometer.

- 20     Fig. 6 shows the predicted lengths of domain I amplification products of rat  $\alpha$ -subunits and their subunit-specific restriction enzyme profile.

Figs. 7A-7B set forth the nucleotide and amino acid sequences of the murine *NaN*.

- Fig. 8A-Fig.8B. Fig.8A is a partial nucleotide sequence of the human *NaN*.  
25 Fig. 8B is a partial amino acid sequence of the human *NaN* protein.

Fig. 9 shows cultures of DRG neurons obtained from L4/5 ganglia of adult rats that were reacted with antibody to *NaN* and then processed for immunofluorescent localization. a.,b. *NaN* immunostaining is prominent within the cell bodies of DRG neurons. c. *NaN* is present in the neuritic outgrowths, as

well as the cell bodies, of DRG neurons. d., d'. Nomarski (d.) and fluorescent (d'.) images of a neuron that does not express NaN protein.

Fig. 10 shows the location of *Scn1 la* and related genes on distal mouse chromosome 9. (A) Haplotypes from the Jackson BSS backcross. Black boxes represent C57BL/6J alleles and white boxes represent SPRET/Ei alleles. The number of animals with each haplotype is given below each column. Missing data was inferred from adjacent data when typing was ambiguous. (B) Map of distal chromosome 9 based on data in (A). Positions of *Scn5a* and *Scn10a* from the MGD consensus map and the locations of the human orthologs are indicated. Numbers are cM positions on the consensus map (<http://www.informatics.jax.org/bin/ccr/index>).

#### DETAILED DESCRIPTION

The present invention relates to a novel gene that we have discovered, called *NaN*. *NaN* encodes a previously unidentified protein, referred to herein as NaN, that belongs to the  $\alpha$ -subunit voltage-gated sodium channel protein family and that produces a TTX-R sodium current. Such channels underlie the generation and propagation of impulses in excitable cells like neurons and muscle fibers. *NaN* is a novel sodium channel, with a sequence distinct from other, previously identified, channels. The preferential expression of *NaN* on sensory, but not other neurons, makes it a very useful target for diagnostic and/or therapeutic uses in relation to acute and/or chronic pain pathologies.

#### Definitions:

This specification uses several technical terms and phrases which are intended to have the following meanings:

The phrase "modulate" or "alter" refers to up- or down-regulating the level or activity of a particular receptor, ligand or current flow. For example an agent might modulate  $\text{Na}^+$  current flow by inhibiting (decreasing) or enhancing (increasing)  $\text{Na}^+$  current flow. Similarly, an agent might modulate the level of

expression of the Na<sup>+</sup> sodium channel or the activity of the Na<sup>+</sup> channels that are expressed.

The phrase "sodium current" or "Na<sup>+</sup> current" means the flow of sodium ions across a cell membrane, often through channels (specialized protein molecules) that are specifically permeable to certain ions, in this case sodium ions.

The phrase "voltage gated" means that the ion channel opens when the cell membrane is in a particular voltage range. Voltage-sensitive sodium channels open when the membrane is depolarized. They then permit Na<sup>+</sup> ions to flow into the cell, producing further depolarization. This permits the cell to generate electrical impulses (also known as "action potentials").

The phrase "rapidly repriming" means that the currents recover from inactivation more rapidly than do such currents in most other voltage gated sodium channel family members.

The terms "TTX-R" and "TTX-S" means that the flow of current through a cell membrane is, respectively, resistant or sensitive to tetrodotoxin (a neurotoxin produced in certain species) at a concentration of about 100 nM.

The phrase "peripheral nervous system (PNS)" means the part of the nervous system outside of the brain and spinal cord, *i.e.*, the spinal roots and associated ganglia such as dorsal root ganglia (DRG) and trigeminal ganglia, and the peripheral nerves.

The phrase "inhibits Na<sup>+</sup> current flow" means that an agent has decreased such current flow relative to a control cell not exposed to that agent. A preferred inhibitor will selectively inhibit such current flow, without affecting the current flow of other sodium channels; or it will inhibit Na<sup>+</sup> current in the channel of interest to a much larger extent than in other channels.

The phrase "enhances Na<sup>+</sup> current flow" means that an agent has increased such current flow relative to a control cell not exposed to that agent. A preferred agent will selectively increase such current flow, without affecting

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the current flow of other sodium channels; or it will increase  $\text{Na}^+$  current in the channel of interest to a much larger extent than in other channels.

The phrase "specifically hybridizes" refers to nucleic acids which hybridize under highly stringent or moderately stringent conditions to the  
5 nucleic acids encoding the NaN sodium channel, such as the DNA sequence of Figs. 1, 7A or 8A.

The phrase "isolated nucleic acid" refers to nucleic acids that have been separated from or substantially purified relative to contaminant nucleic acids encoding other polypeptides. "Nucleic acids" refers to all forms of DNA and  
10 RNA, including cDNA molecules and antisense RNA molecules.

The phrase "RT-PCR" refers to the process of reverse transcription of RNA (RT) using the enzyme reverse transcriptase, followed by the amplification of certain cDNA templates using the polymerase chain reaction (PCR); PCR requires generic or gene-specific primers and thermostable DNA  
15 polymerase, for example, *Taq* DNA polymerase.

The phrase "preferentially expressed" means that voltage gated  $\text{Na}^+$  channel is expressed in the defined tissues in detectably greater quantities than in other tissues. For instance, a voltage gated  $\text{Na}^+$  channel that is preferentially expressed in dorsal root ganglia or trigeminal ganglia is found in detectably  
20 greater quantities in dorsal root ganglia or trigeminal ganglia when compared to other tissues or cell types. The quantity of the voltage gated  $\text{Na}^+$  channel may be detected by any available means, including the detection of specific RNA levels and detection of the channel protein with specific antibodies.

Characterization of the NaN Sodium Channel:

25 The present invention relates to a previously unidentified, voltage-gated sodium channel  $\alpha$ -subunit (*NaN*), predicted to be TTX-R, voltage-gated, and preferentially expressed in sensory neurons innervating the body (dorsal root ganglia or DRG) and the face (trigeminal ganglia). The predicted open reading frame (ORF), the part of the sequence coding for the NaN protein molecule, has

been determined with the putative amino acid sequence from different species (rat, mouse, human) presented in Figs. 2, 7B and 8B.

All of the relevant landmark sequences of voltage-gated sodium channels are present in NaN at the predicted positions, indicating that *NaN* belongs to the sodium channel family. But NaN is distinct from all other previously identified Na channels, sharing a sequence identity of less than 53% with each one of them. *NaN* is distinct from *SNS*, the only other TTX-R Na<sup>+</sup> channel subunit that has been identified, until our discovery, in PNS. We have identified and cloned *NaN* without using any primers or probes that are based upon or specific to *SNS*. Moreover, *NaN* and *SNS* share only 47% similarity of their predicted open reading frame (ORF), comparable to the limited similarity of *NaN* to all subfamily 1 members.

The low sequence similarity to existing  $\alpha$ -subunits clearly identifies *NaN* as a novel gene, not simply a variant of an existing channel. Sequence variations compared to the other voltage-gated channels indicate that NaN may be the prototype of a novel and previously unidentified, third class of TTX-R channels that may possess distinct properties compared to *SNS*. NaN and *SNS*, which are present in nociceptive DRG and trigeminal neurons, may respond to pharmacological interventions in different ways. The preferential expression of NaN in sensory DRG and trigeminal neurons provides a target for selectively modifying the behavior of these nerve cells while not affecting other nerve cells in the brain and spinal cord. A further elucidation of the properties of NaN channels will be important to understand more fully the effects of drugs designed to modulate the function of the "TTX-R" currents which are characteristic of DRG nociceptive neurons and which contribute to the transmission of pain messages, and to abnormal firing patterns after nerve injury and in other painful conditions.

#### *NaN Nucleic Acids:*



Nucleic acid molecules of the invention include the nucleotide sequences set forth in Fig. 1, Fig. 7A, Fig. 8A as well as nucleotide sequences that encode the amino acid sequences of Fig. 2, Fig. 7B and Fig. 8B. Nucleic acids of the claimed invention also include nucleic acids which specifically

5 hybridize to nucleic acids comprising the nucleotide sequences set forth in Fig. 1, Fig. 7A or 8A or nucleotide sequences which encode the amino acid sequences of Fig. 2, Fig. 7B or Fig. 8B. A nucleic acid which specifically hybridizes to a nucleic acid comprising that sequence remains stably bound to said nucleic acid under highly stringent or moderately stringent conditions.

10 Stringent and moderately stringent conditions are those commonly defined and available, such as those defined by Sambrook *et al.* [59] or Ausubel *et al.* [60]. The precise level of stringency is not important, rather, conditions should be selected that provide a clear, detectable signal when specific hybridization has occurred.

15 Hybridization is a function of sequence identity (homology), G+C content of the sequence, buffer salt content, sequence length and duplex melt temperature ( $T_m$ ) among other variables. See, Maniatis *et al.* [62]. With similar sequence lengths, the buffer salt concentration and temperature provide useful variables for assessing sequence identity (homology) by hybridization

20 techniques. For example, where there is at least 90 percent homology, hybridization is commonly carried out at 68° C in a buffer salt such as 6XSSC diluted from 20XSSC. See Sambrook *et al.* [59]. The buffer salt utilized for final Southern blot washes can be used at a low concentration, *e.g.*, 0.1XSSC and at a relatively high temperature, *e.g.*, 68° C, and two sequences will form a

25 hybrid duplex (hybridize). Use of the above hybridization and washing conditions together are defined as conditions of high stringency or highly stringent conditions. Moderately stringent conditions can be utilized for hybridization where two sequences share at least about 80 percent homology. Here, hybridization is carried out using 6XSSC at a temperature of about

30 50-55° C. A final wash salt concentration of about 1-3XSSC and at a

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temperature of about 60-68° C are used. These hybridization and washing conditions define moderately stringent conditions.

In particular, specific hybridization occurs under conditions in which a high degree of complementarity exists between a nucleic acid comprising the  
5 sequence of an isolated sequence and another nucleic acid. With specific hybridization, complementarity will generally be at least about 70%, 75%, 80%, 85%, preferably about 90-100%, or most preferably about 95-100%.

As used herein, homology or identity is determined by **BLAST** (Basic Local Alignment Search Tool) analysis using the algorithm employed by the  
10 programs **blastp**, **blastn**, **blastx**, **tblastn** and **tblastx** (Karlin *et al.* Proc. Natl. Acad. Sci. USA 87: 2264-2268 (1990) and Altschul, S. F. J. Mol. Evol. 36: 290-300(1993), both of which are herein incorporated by reference) which are tailored for sequence similarity searching. The approach used by the **BLAST** program is to first consider similar segments between a query sequence and a  
15 database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al.* (Nature Genetics 6: 119-129 (1994)) which is herein incorporated by reference. The search  
20 parameters for **histogram**, **descriptions**, **alignments**, **expect** (i.e., the statistical significance threshold for reporting matches against database sequences), **cutoff**, **matrix** and **filter** are at the default settings. The default scoring matrix used by **blastp**, **blastx**, **tblastn**, and **tblastx** is the **BLOSUM62** matrix (Henikoff *et al.* Proc. Natl. Acad. Sci. USA 89: 10915-10919 (1992), herein  
25 incorporated by reference). For **blastn**, the scoring matrix is set by the ratios of **M** (i.e., the reward score for a pair of matching residues) to **N** (i.e., the penalty score for mismatching residues), wherein the default values for **M** and **N** are 5 and -4, respectively.

The nucleic acids of the present invention can be used in a variety of  
30 ways in accordance with the present invention. For example, they can be used

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- as nucleic acid probes to screen other cDNA and genomic DNA libraries so as to select by hybridization other DNA sequences that encode homologous *NaV* sequences. Contemplated nucleic acid probes could be RNA or DNA labeled with radioactive nucleotides or by non-radioactive methods (for example, 5 biotin). Screening may be done at various stringencies (through manipulation of the hybridization  $T_m$ , usually using a combination of ionic strength, temperature and/or presence of formamide) to isolate close or distantly related homologs. The nucleic acids may also be used to generate primers to amplify cDNA or genomic DNA using polymerase chain reaction (PCR) techniques.
- 10 The nucleic acid sequences of the present invention can also be used to identify adjacent sequences in the genome, for example, flanking sequences and regulatory elements of *NaV*. The nucleic acids may also be used to generate antisense primers or constructs that could be used to modulate the level of gene expression of *NaN*. The amino acid sequence may be used to design and
- 15 produce antibodies specific to *NaN* that could be used to localize *NaN* to specific cells and to modulate the function of *NaN* channels expressed on the surface of cells.

Vectors and Transformed Host Cells:

- The present invention also comprises recombinant vectors containing and 20 capable of replicating and directing the expression of nucleic acids encoding a *NaN* sodium channel in a compatible host cell. For example, the insertion of a DNA in accordance with the present invention into a vector using enzymes such as T4 DNA ligase, may be performed by any conventional means. Such an insertion is easily accomplished when both the DNA and the desired vector
- 25 have been cut with the same restriction enzyme or enzymes, since complementary DNA termini are thereby produced. If this cannot be accomplished, it may be necessary to modify the cut ends that are produced by digesting back single-stranded DNA to produce blunt ends, or by achieving the same result by filling in the single-stranded termini with an appropriate DNA

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polymerase. In this way, blunt-end ligation may be carried out. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini. Such linkers may comprise specific oligonucleotide sequences that encode restriction site recognition sequences.

- 5           Any available vectors and the appropriate compatible host cells may be used [59, 60]. Commercially available vectors, for instance, those available from New England Biolabs Inc., Promega Corp., Stratagene Inc. or other commercial sources are included.

- The transformation of appropriate cell hosts with an rDNA (recombinant  
10 DNA) molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. Frog oocytes can be injected with RNA and will express channels, but in general, expression in a mammalian cell line (such as HEK293 or CHO cells) is preferred. With regard to transformation of prokaryotic host cells,  
15 electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.* [61]; and [62]. With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment methods are typically employed [63, 64].

- Successfully transformed cells, *i.e.*, cells that contain an rDNA molecule  
20 of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using conventional methods [65, 66] or the proteins produced from the  
25 cell assayed via an immunological method. If tags such as green fluorescent protein are employed in the construction of the recombinant DNA, the transfected cells may also be detected *in vivo* by the fluorescence of such molecules by cell sorting.

- For transient expression of recombinant channels, transformed host cells  
30 for the measurement of Na<sup>+</sup> current or intracellular Na<sup>+</sup> levels are typically

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prepared by co-transfecting constructs into cells such as HEK293 cells with a fluorescent reporter plasmid (such as pGreen Lantern-1, Life Technologies, Inc.) using the calcium-phosphate precipitation technique [27]. HEK293 cells are typically grown in high glucose DMEM (Life Technologies, Inc)

5 supplemented with 10% fetal calf serum (Life Technologies, Inc). After 48 hrs, cells with green fluorescence are selected for recording [28].

For preparation of cell lines continuously expressing recombinant channels, the *NaV* construct is cloned into other vectors that carry a selectable marker in mammalian cells. Transfections are carried out using the calcium

10 phosphate precipitation technique [27]. Human embryonic kidney (HEK-293), chinese hamster ovary (CHO) cells, derivatives of either or other suitable cell lines are grown under standard tissue culture conditions in Dulbeccos's modified Eagle's medium supplemented with 10% fetal bovine serum. The calcium phosphate-DNA mixture is added to the cell culture medium and left for 15-20

15 hr, after which time the cells are washed with fresh medium. After 48 hrs, antibiotic (G418, Geneticin, Life Technologies) is added to select for cells which have acquired neomycin resistance. After 2-3 weeks in G418, 10-20 isolated cell colonies are harvested using sterile 10ml pipette tips. Colonies are grown for another 4-7 days, split and subsequently tested for channel expression

20 using whole-cell patch-clamp recording techniques and RT-PCR.

Method of Measuring  $Na^+$  Current Flow:

$Na^+$  currents are measured using patch clamp methods [29], as described by Rizzo *et al.* [30] and Dib-Hajj *et al.* [28]. For these recordings data are acquired on a MacIntosh Quadra 950 or similar computer, using a program such

25 as Pulse (v 7.52, HEKA, German). Fire polished electrodes typically (0.8-1.5 MW) are fabricated from capillary glass using a Sutter P-87 puller or a similar instrument. In the most rigorous analyses, cells are usually only considered for analysis if initial seal resistance is  $<5$  Gohm, they have high leakage currents (holding current  $<0.1$  nA at  $-80$  mV), membrane blebs, and an access resistance

<5 Mohm. Access resistance is usually monitored throughout the experiment and data are not used if resistance changes occur. Voltage errors are minimized using series resistance compensation and the capacitance artifact is canceled using computer controlled amplifier circuitry or other similar methods. For  
5 comparisons of the voltage dependence of activation and inactivation, cells with a maximum voltage error of  $\pm 10$  mV after compensation are used. Linear leak subtraction is usually used for voltage clamp recordings. Membrane currents are typically filtered at 5 KHz and sampled at 20 KHz. The pipette solution contains a standard solution such as: 140 mM CsF, 2 mM MgCl<sub>2</sub>, 1 mM EGTA,  
10 and 10 mM Na-HEPES (pH 7.3). The standard bathing solution is usually 140 mM NaCl, 3 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10 mM HEPES, and 10 mM glucose (pH 7.3).

Voltage clamp studies on transformed cells or DRG neurons, using methods such as intracellular patch-clamp recordings, can provide a quantitative  
15 measure of the sodium current density (and thus the number of sodium channels in a cell), and channel physiological properties. These techniques, which measure the currents that flow through ion channels such as sodium channels, are described in Rizzo *et al.* [21]. Alternatively, the blockage or enhancement of sodium channel function can be measured using optical imaging with  
20 sodium-sensitive dyes or with isotopically labeled Na. These methods which are described in Rose, *et al.*, (*J. Neurophysiology*, 1997 in press) [67] and by Kimelberg and Walz [31], measure the increase in intracellular concentration of sodium ions that occurs when sodium channels are open.

#### Measurement of Intracellular Sodium ( $[Na^+]_i$ )

25 The effects of various agents on cells that express Na<sup>+</sup> can be determined using ratiometric imaging of  $[Na^+]_i$  using SBFI or other similar ion-sensitive dyes. In this method, as described by Sontheimer *et al.* [32], cytosolic-free Na<sup>+</sup> is measured using an indicator for Na<sup>+</sup>, such as SBFI (sodium-binding benzofuran isophthalate; [33]) or a similar dye. Cells are first loaded with the

membrane-permeable acetoxymethyl ester form of the dye (which is dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM). Recordings are obtained on the stage of a microscope using a ratiometric imaging setup (e.g., from Georgia Instruments). Excitation light is provided at appropriate wavelengths (e.g., 340:385 nm). Excitation light is passed to the cells through a dichroic reflector (400 nm) and emitted light above 450 nm is collected. Fluorescence signals are amplified, e.g., by an image intensifier (GenIISyS) and collected with a CCD camera, or similar device, interfaced to a frame grabber. To account for fluorescence rundown, the fluorescence ratio 340:385 is used to assay cytosolic-free  $\text{Na}^+$ .

For calibration of SBFI's fluorescence, cells are perfused with calibration solutions containing known  $\text{Na}^+$  concentrations (typically 0 and 30 mM, or 0, 30, and 50 mM  $[\text{Na}^+]$ ), and with ionophores such as gramicidin and monensin (see above) after each experiment. As reported by Rose and Ransom [34], the 345/390 nm fluorescence ratio of intracellular SBFI changes monotonically with changes in  $[\text{Na}^+]$ . Experiments are typically repeated on multiple (typically at least 4) different coverslips, providing statistically significant measurements of intracellular sodium in control cells, and in cells exposed to various concentrations of agents that may block, inhibit or enhance  $\text{Na}^+$ .

Method to Measure  $\text{Na}^+$  Influx via Measuring  $^{22}\text{Na}$  or  $^{86}\text{Rb}$ .

$^{22}\text{Na}$  is a gamma emitter and can be used to measure  $\text{Na}^+$  flux [31], and  $^{86}\text{Rb}^+$  can be used to measure  $\text{Na}^+/\text{K}^+$ -ATPase activity [32].  $^{86}\text{Rb}^+$  ions are taken up by the  $\text{Na}^+/\text{K}^+$ -ATPase-like  $\text{K}^+$  ions, but have the advantage of a much longer half-life than  $^{42}\text{K}^+$  [35]. Thus, measurement of the unidirectional ouabain-sensitive  $^{86}\text{Rb}^+$  uptake provides a quantitative method for assaying  $\text{Na}^+/\text{K}^+$ -ATPase activity which provides another indicator of the electrical firing of nerve cells. Following incubation of cells expressing *NaN* with the isotope  $^{22}\text{Na}^+$ , the cellular content of the isotope is measured by liquid scintillation

counting or a similar method, and cell protein is determined using a method such as the bicinchoninic acid protein assay [36] following the modifications described by Goldschmidt and Kimelberg [37] for cultured cells.  $^{22}\text{Na}$  and  $^{86}\text{Rb}^+$  fluxes are determined in the presence and absence of agents that may block, inhibit, or enhance NaN. This permits determination of the actions of these agents on NaN.

Method to Identify Agents that Modulate NaN-Mediated Current:

Several approaches can be used to identify agents that are able to modulate (i.e., block or augment) the  $\text{Na}^+$  current through the NaN sodium channel. In general, to identify such agents, a model cultured cell line that expresses the NaN sodium channel is utilized, and one or more conventional assays are used to measure  $\text{Na}^+$  current. Such conventional assays include, for example, patch clamp methods, the ratiometric imaging of  $[\text{Na}^+]_i$ , and the use of  $^{22}\text{Na}$  and  $^{86}\text{Rb}$  as described above.

In one embodiment of the present invention, to evaluate the activity of a candidate compound to modulate  $\text{Na}^+$  current, an agent is brought into contact with a suitable transformed host cell that expresses NaN. After mixing or appropriate incubation time, the  $\text{Na}^+$  current is measured to determine if the agent inhibited or enhanced  $\text{Na}^+$  current flow.

Agents that inhibit or enhance  $\text{Na}^+$  current are thereby identified. A skilled artisan can readily employ a variety of art-recognized techniques for determining whether a particular agent modulates the  $\text{Na}^+$  current flow.

Because  $\text{Na}^+$  is preferentially expressed in pain-signaling cells, one can also design agents that block, inhibit, or enhance  $\text{Na}^+$  channel function by measuring the response of laboratory animals, treated with these agents, to acute or chronic pain. In one embodiment of this aspect of the invention, laboratory animals such as rats are treated with an agent for instance, an agent that blocks or inhibits (or is thought to block or inhibit) NaN. The response to various painful stimuli are then measured using tests such as the tail-flick test and limb



withdrawal reflex, and are compared to untreated controls. These methods are described in Chapter 15 of Reference [38]. In another embodiment of this aspect of the invention, laboratory animals such as rats are subjected to localized injection of pain-producing inflammatory agents such as formalin

5 [39], Freund's adjuvant [40] or carageenan, or are subjected to nerve constriction [41,42] or nerve transection [43] which produce persistent pain. The response to various normal and painful stimuli are then measured, for example, by measuring the latency to withdrawal from a warm or hot stimulus [38] so as to compare control animals and animals treated with agents that are thought to

10 modify NaN.

The preferred inhibitors and enhancers of NaN preferably will be selective for the NaN  $\text{Na}^+$  channel. They may be totally specific (like tetrodotoxin, TTX, which inhibits sodium channels but does not bind to or directly effect any other channels or receptors), or relatively specific (such as

15 lidocaine which binds to and blocks several types of ion channels, but has a predilection for sodium channels). Total specificity is not required for an inhibitor or enhancer to be efficacious. The ratio of its effect on sodium channels vs. other channels and receptors, may often determine its effect and effects on several channels, in addition to the targeted one, may be efficacious

20 [44].

It is contemplated that modulating agents of the present invention can be, as examples, peptides, small molecules, naturally occurring and other toxins and vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the modulating

25 agents of the present invention. Screening of libraries of molecules may reveal agents that modulate NaN or current flow through it. Similarly, naturally occurring toxins (such as those produced by certain fish, amphibians and invertebrates) can be screened. Such agents can be routinely identified by exposing a transformed host cell or other cell which expresses a sodium channel

30 to these agents and measuring any resultant changes in  $\text{Na}^+$  current

Recombinant Protein Expression, Synthesis and Purification:

Recombinant NaN proteins can be expressed, for example, in *E. coli* strains HB101, DH5a or the protease deficient strain such as CAG-456 and purified by conventional techniques.

- 5           The peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems. The production  
10 using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

Antibodies and Immunodetection:

- Another class of agents of the present invention are antibodies immunoreactive with the Na<sup>+</sup> channel. These antibodies may block, inhibit, or  
15 enhance the Na<sup>+</sup> current flow through the channel. Antibodies can be obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of NaN, particularly (but not necessarily) those that are exposed extracellularly on the cell surface. Such immunological agents also can be used in competitive binding studies to identify second generation  
20 inhibitory agents. The antibodies may also be useful in imaging studies, once appropriately labeled by conventional techniques.

Production of Transgenic Animals:

- Transgenic animals containing and mutant, knock-out or modified NaN genes are also included in the invention. Transgenic animals wherein both NaN  
25 and the SNS/PN3 gene are modified, disrupted or in some form modified are also included in the present invention. Transgenic animals are genetically modified animals into which recombinant, exogenous or cloned genetic material has been experimentally transferred. Such genetic material is often referred to

as a "transgene". The nucleic acid sequence of the transgene, in this case a form of *NaN*, may be integrated either at a locus of a genome where that particular nucleic acid sequence is not otherwise normally found or at the normal locus for the transgene. The transgene may consist of nucleic acid sequences derived  
5 from the genome of the same species or of a different species than the species of the target animal.

The term "germ cell line transgenic animal" refers to a transgenic animal in which the genetic alteration or genetic information was introduced into a germ line cell, thereby conferring the ability of the transgenic animal to transfer  
10 the genetic information to offspring. If such offspring in fact possess some or all of that alteration or genetic information, then they too are transgenic animals.

The alteration or genetic information may be foreign to the species of animal to which the recipient belongs, foreign only to the particular individual recipient, or may be genetic information already possessed by the recipient. In  
15 the last case, the altered or introduced gene may be expressed differently than the native gene.

Transgenic animals can be produced by a variety of different methods including transfection, electroporation, microinjection, gene targeting in embryonic stem cells and recombinant viral and retroviral infection (*see, e.g.*,  
20 U.S. Patent No. 4,736,866; U.S. Patent No. 5,602,307; Mullins *et al.* (1993) Hypertension 22(4):630-633; Brenin *et al.* (1997) Surg. Oncol. 6(2)99-110; Tuan (ed.), *Recombinant Gene Expression Protocols*, Methods in Molecular Biology No. 62, Humana Press (1997)).

A number of recombinant or transgenic mice have been produced,  
25 including those which express an activated oncogene sequence (U.S. Patent No. 4,736,866); express simian SV 40 T-antigen (U.S. Patent No. 5,728,915); lack the expression of interferon regulatory factor 1 (IRF-1) (U.S. Patent No. 5,731,490); exhibit dopaminergic dysfunction (U.S. Patent No. 5,723,719); express at least one human gene which participates in blood pressure control  
30 (U.S. Patent No. 5,731,489); display greater similarity to the conditions existing

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in naturally occurring Alzheimer's disease (U.S. Patent No. 5,720,936); have a reduced capacity to mediate cellular adhesion (U.S. Patent No. 5,602,307); possess a bovine growth hormone gene (Clutter *et al.* (1996) Genetics 143(4):1753-1760); or, are capable of generating a fully human antibody  
5 response (McCarthy (1997) The Lancet 349(9049):405).

While mice and rats remain the animals of choice for most transgenic experimentation, in some instances it is preferable or even necessary to use alternative animal species. Transgenic procedures have been successfully utilized in a variety of non-murine animals, including sheep, goats, pigs, dogs,  
10 cats, monkeys, chimpanzees, hamsters, rabbits, cows and guinea pigs (*see, e.g.*, Kim *et al.* (1997) Mol. Reprod. Dev. 46(4):515-526; Houdebine (1995) Reprod. Nutr. Dev. 35(6):609-617; Petters (1994) Reprod. Fertil. Dev. 6(5):643-645; Schnieke *et al.* (1997) Science 278(5346):2130-2133; and Amoah (1997) J. Animal Science 75(2):578-585).

15 The method of introduction of nucleic acid fragments into recombination competent mammalian cells can be by any method which favors co-transformation of multiple nucleic acid molecules. Detailed procedures for producing transgenic animals are readily available to one skilled in the art, including the disclosures in U.S. Patent No. 5,489,743 and U.S. Patent No.  
20 5,602,307.

The specific examples presented below are illustrative only and are not intended to limit the scope of the invention.

## EXAMPLES

Example 1: Cloning and Characterization of the Rat *NaV* Coding  
25 Sequence

### a. RNA Preparation

Dorsal root ganglia (DRG) from the lumbar region (L4-L5) were dissected from adult Sprague-Dawley rats and total cellular RNA was isolated

by the single step guanidinium isothiocyanate-acid phenol procedure [45]. For analytical applications, DRG tissues were dissected from a few animals at a time. The quality and relative yield of the RNA was assessed by electrophoresis in a 1% agarose gel. Because of the limited starting material (4 DRGs weigh on average 10 mg), quantifying the RNA yield was not attempted. PolyA<sup>+</sup> RNA was purified from about 300 µg of total DRG RNA (28 animals) using the PolyAtract isolation system according to the manufacturers recommendations (Promega). Half of the purified RNA was used for the preparation of Marathon cDNA (see below) without further quantification.

10        b. Reverse Transcription

For analytical applications, first strand cDNA was synthesized essentially as previously described [46]. Briefly, total RNA was reverse transcribed in a 25 µl final volume using 1µM random hexamer (Boehringer Mannheim) and 500 units SuperScript II reverse transcriptase (Life Technologies) in the presence of 100 units of RNase Inhibitor (Boehringer Mannheim). The reaction buffer consisted of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT and 125 µM dNTP. The reaction was allowed to proceed at 37°C for 90 min., 42°C for 30 min, then terminated by heating to 65°C for 10 min.

20        c. First-Strand cDNA Synthesis

The Marathon cDNA synthesis protocol was followed according to the manufacturer's instruction as summarized below (all buffers and enzymes are purchased from the manufacturer (Clontech):

Combine the following reagents in a sterile 0.5-ml microcentrifuge tube:

25    1 µg (1-4 µl) PolyA<sup>+</sup> RNA sample, 1 µl cDNA Synthesis Primer (10 µM) and sterile H<sub>2</sub>O to a final volume of 5 µl. Mix contents and spin the tube briefly in a microcentrifuge. Incubate the mixture at 70°C for 2 min., then immediately quench the tube on ice for 2 min. Touch-spin the tube briefly to collect the

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- condensation. Add the following to each reaction tube: 2  $\mu$ l 5X First-Strand Buffer, 1  $\mu$ l dNTP Mix (10 mM), 1  $\mu$ l [ $\alpha$ - $^{32}$ P]dCTP (1  $\mu$ Ci/ $\mu$ l), 1  $\mu$ l AMV Reverse Transcriptase (20 units/ $\mu$ l) for a 10  $\mu$ l volume. The radiolabeled dCTP is optional (used to determine yield of cDNA) and is replaced by sterile H<sub>2</sub>O if
- 5 not used. Mix the contents of the tube by gently pipetting and touch-spin the tube to collect the contents at the bottom. Incubate the mixture at 42°C for 1 hr in an air incubator to reduce condensation and enhance the yield of the first strand cDNA. Place the tube on ice to terminate first-strand synthesis.

d. Second-Strand cDNA Synthesis

- 10 Combine the following components in the reaction tube from above:  
48.4  $\mu$ l Sterile H<sub>2</sub>O, 16  $\mu$ l 5X Second-Strand Buffer, 1.6  $\mu$ l dNTP Mix (10 mM), 4  $\mu$ l 20X Second-Strand Enzyme Cocktail for an 80  $\mu$ l total volume. Mix the contents thoroughly with gentle pipetting and spin the tube briefly in a microcentrifuge. Incubate the mixture at 16°C for 1.5 hr. then add 2  $\mu$ l (10
- 15 units) of T4 DNA Polymerase, mix thoroughly with gentle pipetting and incubate the mixture at 16°C for 45 min. Add 4  $\mu$ l of the EDTA/Glycogen mix to terminate second-strand synthesis. Extract the mixture with an equal volume of buffer-saturated (pH 7.5) phenol:chloroform:isoamyl alcohol (25:24:1). Mix the contents thoroughly by vortexing and spin the tube in a microcentrifuge at
- 20 maximum speed (up to 14,000 rpm or 13000xg), 4°C for 10 min. to separate layers. Carefully transfer the top aqueous layer to a clean 0.5-ml tube. Extract the aqueous layer with 100  $\mu$ l of chloroform:isoamyl alcohol (24:1), vortex, and spin the tube as before to separate the layers. Collect the top layer into a clean 0.5-ml microcentrifuge tube. Ethanol precipitate the double-stranded cDNA by
- 25 adding one-half volume of 4 M Ammonium Acetate and 2.5 volumes of room-temperature 95% ethanol. Mix thoroughly by vortexing and spin the tube immediately in a microcentrifuge at top speed, room temperature for 20 min. Remove the supernatant carefully and wash the pellet with 300  $\mu$ l of 80% ethanol. Spin the tube as before for 10 min. and carefully remove the

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supernatant. Air dry the pellet for up to 10 min. and dissolve the cDNA in 10  $\mu$ l of sterile H<sub>2</sub>O and store at -20°C. Analyze the yield and size of cDNA by running 2  $\mu$ l of the cDNA solution on a 1.2% agarose/EtBr gel with suitable DNA size markers (for example the 1 Kbp ladder, Gibco/BRL). If EtBr staining  
5 does not show a signal and [ $\alpha$ -<sup>32</sup>P]dCTP was included in the reaction, dry the agarose gel on a vacuum gel drying system and expose an x-ray film to the gel overnight at -70°C.

e. Adaptor Ligation

Combine these reagents in a 0.5-ml microcentrifuge test tube, at room  
10 temperature, and in the following order: 5  $\mu$ l ds cDNA, 2  $\mu$ l Marathon cDNA Adaptor (10  $\mu$ M), 2  $\mu$ l 5X DNA Ligation Buffer, 1  $\mu$ l T4 DNA Ligase (1 unit/ $\mu$ l) for a 10  $\mu$ l final volume. Mix the contents thoroughly with gentle pipetting and spin the tube briefly in a microcentrifuge. Incubate at either: 16°C overnight; or room temperature (19-23°C) for 3-4 hr. Inactivate the ligase  
15 enzyme by heating the mixture at 70°C for 5 min. Dilute 1  $\mu$ l of this reaction mixture with 250  $\mu$ l of Tricine-EDTA buffer and use for RACE protocols. Store the undiluted adaptor-ligated cDNA at -20°C for future use.

f. PCR

For the initial discovery of *NaN*, we used generic primers designed  
20 against highly conserved sequences in domain 1 (D1) of  $\alpha$ -subunits I, II and III and later added more primers to accommodate the new  $\alpha$ -subunits that were discovered. Thus, we used generic primers that recognize conserved sequences in all known Na<sup>+</sup> channels. The middle of the amplified region shows significant sequence and length polymorphism (Fig. 6) and [47,48]. Due to  
25 codon degeneracy, 4 forward primers (F1-F4) and 3 reverse primers (R1-R3) were designed to ensure efficient priming from all templates that might have been present in the cDNA pool (Table 1); however, any of these primers may bind to multiple templates depending on the stringency of the reaction. Forward

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primer F1 matches subunits  $\alpha I$ ,  $\alpha III$ ;  $\alpha Na6$ ;  $\alpha PN1$ ;  $\alpha \mu 1$ ,  $\alpha rH1$  and  $\alpha SNS/PN3$ . Sequences of individual subunits show 1 or 2 mismatches to this primer: T to C at position 16 and A to G at position 18 ( $\alpha Na6$ ); C to R at position 6 ( $\alpha \mu 1$ ); A to G at position 18 ( $\alpha rH1$ ) and T to C at position 3 ( $\alpha SNS$ ). Forward primer

5 F2 matches subunit  $\alpha II$ . Forward primer F3 perfectly matches  $\alpha Na6$  and also matches  $\alpha rH1$  with a single mismatch of C to T at position 16. Reverse primer R1 matches subunits  $\alpha I$ ,  $\alpha II$ ,  $\alpha III$ ,  $\alpha Na6$ ,  $\alpha PN1$ ,  $\alpha \mu 1$  and  $\alpha rH1$ . This primer has mismatches compared to 4 subunits: G to A at position 3, A to G at position 4 and T to G at position 7 ( $\alpha I$ ); T to C at position 1 and A to G at

10 position 19 ( $\alpha PN1$ ); G to A at position 3 and A to G at position 7 ( $\alpha \mu 1$ ); an extra G after position 3, GC to CT at positions 14-15, and A to T at position 21 ( $\alpha rH1$ ). Reverse primer R2 matches subunit  $\alpha SNS/PN3$ .



Table 1: Generic and *NaN*-specific primers used for the identification and cloning of *NaN*. All primers except the marathon primers, were synthesized at the department of Pathology, Program for Critical Technologies in Molecular Medicine, Yale University.

5	Forward Primers	Reverse Primers
	1. GACCCRTGGAATTGGTTGGA	1. CAAGAAGGCCCCAGCTGAAGGTGTC
	2. AATCCCTGGAATTGGTTGGA	2. GAGGAATGCCACGCAAAGGAATC
	3. GACCCGTGGAAGTGGTTAGA	3. AAGAAGGGACCAGCCAAAGTTGTC
	4. GATCTTTGGAAGTGGCTTGA	4. ACYTCCATRCANWCCCACAT
10	5. AACATAGTGCTGGAGTTCAGG	5. AGRAARTCNAGCCARCACCA
	6. GTGGCCTTTGGATTCCGGAGG	6. TCTGCTGCCGAGCCAGGTA
	7.	7. CTGAGATAACTGAAATCGCC
	Marathon AP-1 CCATCCTAATACGACTCACTATAGGGC	
	Marathon AP-2 ACTCACTATAGGGCTCGAGCGGC	

15 We used the respective mouse atypical sodium channel mNa.2.3 sequence to design forward primer F4 and reverse primer R3 to amplify the analogous sequence from  $\alpha$ NaG, the presumed rat homolog of mNa.2.3 [14]. The amplified sequence was cloned into the *Srf*I site of the vector pCR-SCRIPT (Stratagene). The nucleotide sequence of this fragment shows

20 88% identity to the respective sequence of mNa.2.3 (Dib-Hajj and Waxman, unpublished [68]). The restriction enzyme *Xba*I was found to be unique to this subunit. Recently, the sequence of a full length cDNA clone of putative sodium channel, NaG-like (SCL-11:Y09164), subunit was published [5]. The published sequence is 99% identical to our sequence and confirms the size and restriction

25 enzyme polymorphism of the NaG PCR product.

The predicted lengths of amplified products and subunit-specific restriction enzyme recognition sites are shown in Fig. 6. All subunit sequences are based on Genbank database (accession numbers:  $\alpha$ I: X03638;  $\alpha$ II: X03639;  $\alpha$ III: Y00766;  $\alpha$ Na6: L39018;  $\alpha$ hNE-Na: X82835;  $\alpha$  $\mu$ 1 M26643;  $\alpha$ rH1 M27902  
5 and  $\alpha$ SNS X92184; mNa 2.3 L36719).

Subsequently, amplification of NaN sequences 3' terminal to the aforementioned fragment was achieved using NaN-specific primers and two generic reverse primers, R4 and R5. The sequence of the R4 primer was based on the amino acid sequence MWV/DCMEV located just N-terminal to domain  
10 II S6 segment (see schematic diagram of Fig. 3 of voltage-gated sodium channel  $\alpha$ -subunits for reference). The sequence of the R5 primer is based on the amino acid sequence AWCWLDFL which forms the N-terminal portion of domain III S3 segment.

Amplification was typically performed in 60  $\mu$ l volume using 1  $\mu$ l of  
15 the first strand cDNA, 0.8mM of each primer and 1.75 units of Expand Long Template DNA polymerase enzyme mixture (Boehringer Mannheim). Compared to conventional and thermostable DNA polymerases, Expand Long Template enzyme mixture increases the yield of the PCR products without an increase in non-specific amplification [49,50]. The PCR reaction buffer  
20 consisted of 50 mM Tris-HCl (pH 9.2), 16 mM  $(\text{NH}_4)_2\text{SO}_4$ , 2.25 mM  $\text{MgCl}_2$ , 2% (v/v) DMSO and 0.1% Tween 20. As described previously [46], amplification was carried out in two stages using a programmable thermal cycler (PTC-200, MJ Research, Cambridge, MA.). First, a denaturation step at 94°C for 4 min, an annealing step at 60°C for 2 min and an elongation step at  
25 72°C for 90 sec. Second, a denaturation step at 94°C for 1 min, an annealing step at 60°C for 1 min and an elongation step at 72°C for 90 sec. The second stage was repeated 33 times for a total of 35 cycles, with the elongation step in the last cycle extended to 10 min.

Primary RACE amplification was performed in 50  $\mu$ l final volume using  
30 4  $\mu$ l diluted DRG marathon cDNA template, 0.2  $\mu$ M marathon AP-1 and

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NaN-specific primers, 3.5 U Expand Long Template enzyme mixture. Extension period was adjusted at 1 min/800 bp based on the expected product. 5' and 3' RACE amplification was performed using primer pairs marathon AP-1/*NaN*-specific R6 and *NaN*-specific F5/marathon AP-1, respectively. The

5 PCR reaction buffer consisted of 50 mM Tris-HCl (pH 9.2), 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3.0 mM MgCl<sub>2</sub>, 2% (v/v) DMSO and 0.1% Tween 20. Amplification in three stages was performed in a programmable thermal cycler (PTC-200, MJ Research, Cambridge, MA.). An initial denaturation step at 92°C was carried out for 2 min. This was followed by 35 cycles consisting of denaturation at

10 92°C for 20 sec, annealing step at 60°C for 1 min, and an elongation step at 68°C. Finally, an elongation step at 68°C was carried out for 5 min. Nested amplification was performed using 2 µl of a 1/500 diluted primary RACE product in a final volume of 50 µl under similar conditions to the primary RACE reactions. Primer pairs AP-2/*NaN*-specific R7 and *NaN*-specific

15 F6/marathon AP-2 were used for nested 5' and 3' RACE, respectively. Secondary RACE products were band isolated from 1% agarose gels and purified using Qiaex gel extraction kit (Qiagen Inc.).

A schematic diagram of the putative structure of *NaN* is shown in Fig.3. The length of the intracellular loops is highly variable both in sequence and

20 length among the various subunits. The exception is the loop between domains III and IV.

**Example 2: Determination of the Putative Rat Amino Acid Sequence for the NaN Channel**

*NaN*-related clones and secondary RACE fragments were sequenced at the

25 W. M. Keck Foundation Biotechnology Resource Lab, DNA sequencing group at Yale University. Sequence analysis including determination of the predicted amino acid sequence was performed using commercial softwares, Lasergene (DNASTAR) and GCG, Inc. The putative amino acid sequence of *NaN* is

shown in Fig. 2. Predicted transmembrane segments of domains I - IV are underlined.

Example 3: Determination of the Murine NaN Sequence

Total RNA extraction from trigeminal ganglia of mice, purification of polyA<sup>+</sup> RNA, and Marathon cDNA construction were done as previously described for the rat. The initial amplification was performed using rat *NaN* primers. The forward primer corresponds to nucleotides 765-787 of the rat sequence (5' CCCTGCTGCGCTCGGTGAAGAAG 3'), and the reverse primer corresponds to nucleotides 1156-1137 (negative strand) of the rat sequence (5' GACAAAGTAGATCCCAGAGG 3'). The amplification produced a fragment of the expected size. The sequence of this fragment demonstrated high similarity to rat *NaN*. Other fragments were amplified using different rat primers and primers designed based on the new mouse *NaN* sequence that was being produced. Finally, longer fragments were amplified using mouse Marathon cDNA template and mouse *NaN*-specific primers in combination with adaptor primers that were introduced during the Marathon cDNA synthesis. These fragments were sequenced using primer walking and assembled into Figure 7A.

Mouse *NaN* nucleotide sequence, like rat *NaN*, lacks the out-of-frame ATG at the -8 position relative to the translation initiation codon ATG at position 41 (Fig. 7A). Translation termination codon TGA is at position 5314. A polyadenylation signal (AATAAA) is present at position 5789 and a putative 23 nucleotide polyA tail is present beginning at position 5800. The sequence encodes an ORF of 1765 a.a. (Fig. 7B), which is 90% similar to rat *NaN*. The gene encoding *NaN* has been named *Scn11a*.

*Chromosomal localization of mouse Nan*

A genetic polymorphism between strains C57BL/6J and SPRET/Ei was identified by SSCP analysis of a 274 bp fragment from the 3'UTR of *Scn11a*.

Genotyping of 94 animals from the BSS backcross panel (Rowe *et al.*, 1994) demonstrated linkage of *Scn11a* with markers on distal chromosome 9 (Fig. 10). No recombinants were observed between *Scn11a* and the microsatellite marker *D9Mit19*. Comparison of our data with the MGD consensus map of mouse chromosome 9 revealed close linkage of *Scn11a* with the two other TTX-R voltage-gated sodium channels, *Scn5a* (George *et al.*, 1995; Klocke *et al.*, 1992) and *Scn10a* (Kozak and Sangameswaran, 1996; Souslova *et al.*, 1997).

Example 4: Determination of a Partial Human NaN Sequence

Human DRG tissue was obtained from a transplant donor. Total RNA extraction and cDNA synthesis were performed as described previously.

Forward primer corresponds to sequence 310-294 (minus strand) of EST AA446878. The sequence of the primer is 5' CTCAGTAGTTGGCATGC 3'. Reverse primer corresponds to sequence 270-247 (minus strand) of EST AA88521 1. The sequence of the primer is 5'GGAAAGAAGCACGACCACACAGTC 3'. Amplification was performed as previously described. PCR amplification was successful and a 2.1 Kbp fragment was obtained. This fragment was gel purified and sent for sequencing by primer walking, similar to what is done for mouse *NaN*. The sequence of the ESTs is extended in both directions; the additional sequence shows highest similarity to rat and mouse *NaN*, compared to the rest of the subunits.

The sequence of a human 2.1 kbp fragment was obtained using the PCR forward and reverse primers for sequencing from both ends of the fragment. Two additional primers were used to cover the rest of the sequence. The sequence was then extended in the 5' direction using forward primer 1 (above) and human *NaN* reverse primer (5'-GTGCCGTAAACATGAGACTGTGCG3') near the 5' end of the 2.1 kb fragment. The partial amino acid sequence is set forth in Figure 8B.

The partial ORF of the human *NaN* consists 1241 amino acids. The sequence is 64% identical to the corresponding sequence of rat *NaN* (73%

similar, allowing for conservative substitutions) using the advanced BLAST program at NIH. Using the Clustal method of alignment (Lasergene software, DNASTar, Inc.) the human NaN is 68% and 69% similar to mouse and rat NaN, respectively. The respective mouse and rat sequences are 88% similar.

5           Example 5: Isolation of an Alternative Splicing Variant of Rat NaN

A rat NaN cDNA that encodes a C-terminal truncated version of the full-length rat NaN in Figures 1 and 2 was isolated by sequencing the insert of a rat cDNA clone. The variant NaN cDNA encodes an NaN protein lacking the 387 C-terminal amino acids of the full length NaN and containing a novel 94 amino acid stretch at the C-terminal end. The new sequence arises from the use of a cryptic donor splice site in exon 23 and a novel exon 23', which is located in intron 23. The novel C terminal amino acids are: AAGQAMRKQG  
10           DILGPNIHQF SQSSETPFLG CPQQRTCVSF VRPQRVLRVP  
              WFPAWRTVTF LSRPRSSESS AWLGLVESSG WSGLPGESGP SSLL. The  
15           N-terminal amino acids of the truncated variant are identical to amino acids 1-1378 of the full length rat NaN of Figure 2. The alternative exon and the splicing pattern was confirmed by comparing the cDNA sequence and the genomic sequence in the respective region.

20           Example 6: Methods to Isolate Other NaN Sequences

a. Isolation of NaN sequences from genomic DNA

The genomic structure of 3 voltage-gated Na<sup>+</sup> channel  $\alpha$ -subunits have already been determined [51-54]. These genes bear remarkable similarity in  
25           their organization and provide a predictable map of most of the exon/intron boundaries. Based on the available rat, mouse and human cDNA sequence of NaN, disclosed herein, PCR primers are designed to amplify NaN homologous sequences from other species using standard PCR protocols.

Alternatively, commercially available genomic DNA libraries are screened with *NaN*-specific probes (based on the rat, mouse, or more preferably, the human sequence) using standard library screening procedures [59, 60]. This strategy yields genomic DNA isolates that can then be sequenced and the exon/intron boundaries determined by homology to the rat, mouse or human cDNA sequence.

b. Isolation of full length NaN sequences from human autopsy or biopsy/surgical tissues

b.1. Isolation of human ganglia total RNA

10 A full length *NaN* human cDNA homologue is isolated from human dorsal root ganglia or trigeminal ganglia or other cranial ganglia from post-mortem human material, fetuses or biopsy or surgical tissues. Total ribonucleic acid (RNA) is isolated from these tissues by extraction in guanidinium isothiocyanate [69] as described in Example 1.

15 b.2 Determination of the full length transcript size of the human homologue of the rat NaN sodium channel cDNA.

The method of determining transcript size is as described in Example 9.

Example 7: Production of human DRG cDNA library

A cDNA library from human DRG or trigeminal ganglia polyA<sup>+</sup> RNA was prepared in Example 4 using standard molecular biology techniques [59, 60].

PolyA<sup>+</sup> mRNA is hybridized to an oligo(dT) primer and the RNA is copied by reverse transcriptase into single strand cDNA. Then, the RNA in the RNA-DNA hybrid is fragmented by RNase H as *E. coli* DNA polymerase I synthesizes the second-strand fragment. The ends of the double stranded cDNA are repaired, linkers carrying specific restriction enzyme site (for example, Eco

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RI) are ligated to the ends using *E. coli* DNA ligase. The pool of the cDNA insert is then ligated into one of a variety of bacteriophage vectors that are commercially available like Lambda-Zap (Stratagene). The procedures are summarized in more detail as follows:

5           a. First strand cDNA Synthesis

Dissolve 10 µg poly(A) + RNA at a concentration of 1 µg/µl in sterile water. Heat the RNA for 2-5 min. at 65°C-70°C, then quench immediately on ice. In a separate tube add in the following order (180 µl total) : 20 µl 5 mM dNTPs (500 uM final each), 40 µl 5x RT buffer (1x final), 10 µl 200 mM DTT  
10 (10 mM final), 20 µl 0.5 mg/ml oligo (dT)12-18 (50 µg/ml final), 60 µl H<sub>2</sub>O, 10 µl (10 U) RNasin (50 U/ml final). Mix by vortexing, briefly microcentrifuge, and add the mixture to the tube containing the RNA. Add 20 µl (200 U) AMV or MMLV reverse transcriptase for a final concentration of 1000 U/ml in 200 µl. Mix by pipetting up and down several times and remove  
15 10 µl to a separate tube containing 1 µl of α<sup>32</sup>P dCTP. Typically, incubate both tubes at room temperature for 5 min., then place both tubes at 42°C for 1.5 hr. This radiolabeled aliquot is removed to determine incorporation and permit an estimation of recovery; this reaction is stopped by adding 1 µl of 0.5 M EDTA, pH 8.0, and stored frozen at -20°C. The radiolabeled reaction will be  
20 used later to estimate the yield and average size of the cDNA inserts. The main reaction is stopped by adding 4 µl of 0.5 M EDTA, pH 8.0, and 200 µl buffered phenol. The mixture is vortexed well, microcentrifuged at room temperature for 1 min. to separate phases, and the upper aqueous layer is transferred to a fresh tube. Back extract the phenol layer with 1X TE buffer (10  
25 mM Tris, 1 mM EDTA, pH 7.5) and pool the aqueous layers from the two extractions. This back extraction of the phenol layer improves the yield. The cDNA is ethanol precipitated using 7.5 M ammonium acetate (final concentration 2.0 to 2.5 M) and 95% ethanol. Place in dry ice/ethanol bath 15 min., warm to 4°C, and microcentrifuge at 10 min. at full speed, 4°C, to pellet



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nucleic acids. The small, yellow-white pellet is then washed with ice-cold 70% ethanol, and microcentrifuged for 3 min. at full speed, 4°C. Again, remove the supernatant, then briefly dry the pellet.

b. Second strand synthesis

- 5 Typically, the pellet from the first-strand synthesis is resuspended in 284  $\mu$ l water and these reagents are added in the following order (400  $\mu$ l total): 4  $\mu$ l 5 mM dNTPs (50 uM final each), 80  $\mu$ l 5x second-strand buffer (1x final), 12  $\mu$ l 5 mM  $\beta$ -NAD (150 uM final), 2  $\mu$ l 10 uCi/ $\mu$ l  $\alpha$ -<sup>32</sup>P dCTP (50 uCi/ml final). Mix by vortexing, briefly microcentrifuge, and add: 4  $\mu$ l (4 U) RNase H  
10 (10 U/ml final), 4  $\mu$ l (20 U) *E. coli* DNA ligase (50 U/ml final), and 10  $\mu$ l (100 U) *E. coli* DNA polymerase I (250 U/ml final). Mix by pipetting up and down, briefly microcentrifuge, and incubate 12 to 16 hr at 14°C. After second-strand synthesis, remove 4  $\mu$ l of the reaction to determine the yield from the incorporation of the radiolabel into acid-insoluble material. Extract the second-  
15 strand synthesis reaction with 400  $\mu$ l buffered phenol and back extract the phenol phase with 200  $\mu$ l TE buffer, pH 7.5, as described above. The double stranded cDNA is then ethanol precipitated as described above.

- To complete the second-strand synthesis the double-stranded cDNA ends are rendered blunt using a mixture of enzymes. Resuspend the pellet in 42  
20  $\mu$ l water then add these reagents in the following order (80  $\mu$ l total) : 5  $\mu$ l 5 mM dNTPs (310 uM final each), 16  $\mu$ l 5x TA buffer (1x final), 1  $\mu$ l 5 mM  $\beta$ -NAD (62 uM final). Mix by vortexing, microcentrifuge briefly, and add: 4  $\mu$ l of 2  $\mu$ g/ml RNase A (100 ng/ml final), 4  $\mu$ l (4 U) RNase H (50 U/ml final), 4  $\mu$ l (20 U) *E. coli* DNA ligase (250 U/ml final), and 4  $\mu$ l (8 U) T4 DNA  
25 polymerase (100 U/ml final). Mix as above and incubate 45 min at 37°C. Add 120  $\mu$ l TE buffer, pH 7.5, and 1  $\mu$ l of 10 mg/ml tRNA. Extract with 200  $\mu$ l buffered phenol and back extract the phenol layer with 100  $\mu$ l TE buffer as described above. Pool the two aqueous layers and ethanol precipitate as described above.

c. Addition of linkers to double stranded cDNA

Combine these reagents in a 0.5-ml microcentrifuge test tube, at room temperature, and in the following order: 100 ng ds cDNA, 2  $\mu$ l linkers/adaptors (10  $\mu$ M), 2  $\mu$ l 5X DNA Ligation Buffer, 1  $\mu$ l T4 DNA Ligase (unit/ $\mu$ l) for a  
5 10  $\mu$ l final volume. Mix the contents thoroughly with gentle pipetting and spin the tube briefly in a microcentrifuge. Incubate at either: 16°C overnight; or room temperature (19-23°C) for 3-4 hr. Inactivate the ligase enzyme by heating the mixture at 70°C for 5 min. This cDNA is typically digested by *Eco* RI to prepare the cohesive ends of the cDNA for ligation into the vector and to cleave  
10 linker concatemers. Typically this reaction consists of the 10  $\mu$ l of the cDNA, 2  $\mu$ l of 10X *Eco* RI buffer (depending on the company of source), 2  $\mu$ l of *Eco* RI (10 units/ $\mu$ l) and sterile H<sub>2</sub>O to a final volume of 20  $\mu$ l. The mixture is incubated at 37°C for 2-4 hrs.

d. Size fractionation of cDNA

15 Size exclusion columns are typically used to remove linker molecules and short cDNA fragments (350 bp). For example, a 1-ml Sepharose CL-4B column is prepared in a plastic column plugged with a small piece of sterilized glass wool (a 5 ml plastic pipet will work fine). The column is equilibrated with 0.1 M sodium chloride in 1x TE (10mM Tris, 1 mM EDTA, pH 7.5). The  
20 cDNA is then loaded onto the column and 200  $\mu$ l fractions are collected. 2  $\mu$ l aliquots of each fraction are analyzed by gel electrophoresis and autoradiography to determine the peak of cDNA elution. Typically, fractions containing the first half of the peak are pooled and purified by ethanol precipitation and resuspending in 10  $\mu$ l distilled water.

e. Cloning of cDNA into bacteriophage vector

Bacteriophage vectors designed for the cloning and propagation of cDNA are provided ready-digested with *Eco* RI and with phosphatased ends from commercial sources (e.g., lambda gt10 from Stratagene). The prepared  
5 cDNA is ligated into lambda vectors following manufacturer's instructions. Ligated vector/cDNA molecules are packaged into phage particles using packaging extracts available commercially.

Example 8: Screening of Human cDNA Library

a. Labeling of cDNA fragments (probes) for library screening

10 An RNA probe is used that recognizes nucleotide sequences that are specific to *NaN*, such as 1371-1751 of *NaN*. Other nucleotide sequences can be developed on the basis of the *NaN* sequence (Fig. 2, 7 and 8) such as nucleotides 765-1160 of the human nucleotide sequence. A *Hind* III/*Bam* HI fragment of *NaN* was inserted in pBluescript (SK+) vector (Stratagene). The  
15 sequence of the resulting construct was verified by sequencing. The orientation of the insert is such that the 5' and 3' ends of the construct delineated by the *Hind* III and *Bam* HI restriction enzyme sites, respectively, are proximal to T7 and T3 RNA polymerase promoters, respectively. Digoxigenin-labeled Sense (linearized at the *Hind* III site and transcribed by T7 RNA polymerase) and  
20 antisense (linearized at the *Bam* HI site and transcribed by T3 RNA polymerase) transcripts were prepared in vitro using MEGAscript transcription kit (Ambion) according to manufacturer specifications. Briefly, 1 µg linearized template was transcribed with the respective RNA polymerase in a 20 µl final volume containing the following reagents: 1X enzyme mixture containing the respective  
25 RNA polymerase and RNase inhibitor and reaction buffer (Ambion), 7.5 mM ATP, GTP and CTP nucleotides, 5.625 mM UTP and 1.725 mM Dig-11UTP (Boehringer Mannheim). In vitro transcription was carried out at 37°C for 3 hrs in a water bath. DNA template was removed by adding 1 µl of RNase-free

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DNase I (2U/ $\mu$ l) to each reaction and incubating further at 37°C for 15 min. The reaction was then stopped by adding 30  $\mu$ l nuclease-free H<sub>2</sub>O and 25  $\mu$ l of LiCl precipitation solution (7.5 M Lithium Chloride, 50 mM EDTA).

The mixture was incubated at -20°C for 30 min. The RNA transcripts  
5 were pelleted in a microfuge at 13000xg, 4°C for 15 min. The supernatant was removed and the pellet washed once with 100  $\mu$ l of 75% ethanol. The mixture was re-centrifuged at 13000xg, room temperature for 5 min. The pellet was then air-dried in a closed chamber and subsequently dissolved in 100  $\mu$ l of RNase-free H<sub>2</sub>O. The transcript yield and integrity were determined by  
10 comparison to a control DIG-labeled RNA on agarose-formaldehyde gel as described in the DIG/Genius kit according to manufacturer recommendations (Boehringer Mannheim). Alternatively, a skilled artisan can design radioactive probes for autoradiographic analysis.

Other regions of the rat, mouse or human *NaV* sodium channel cDNA,  
15 like 3' untranslated sequences, can also be used as probes in a similar fashion for cDNA library screening or Northern blot analysis. Specifically, a probe is made using commercially available kits, such as the Pharmacia oligo labeling kit, or Genius kit (Boehringer Mannheim).

b. cDNA library screening

20 Recombinant plaques containing full length human homologues of the *NaV* sodium channel are detected using moderate stringency hybridization washes (50-60°C, 5 x SSC, 30 minutes), using non-radioactive (see above) or radiolabeled DNA or cRNA *NaV*-specific probes derived from the 3' untranslated or other regions as described above. Libraries are screened using  
25 standard protocols [59, 60] involving the production of nitrocellulose or nylon membrane filters carrying recombinant phages. The recombinant DNA is then hybridized to *NaV*-specific probes (see above). Moderate stringency washes are carried out.

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Plaques which are positive on duplicate filters (*i.e.*, not artefacts or background) are selected for further purification. One or more rounds of screening after dilution to separate the phage are typically performed. Resulting plaques are then purified, DNA is extracted and the insert sizes of these clones

5 characterized. The clones are cross-hybridized to each other using standard techniques [59] and distinct positive clones identified.

Typically, overlapping clones that encode the channel are isolated. Standard cloning techniques are then used to produce a full length cDNA construct that contains any 5' untranslated sequence, the start codon ATG, the

10 coding sequence, a stop codon and any 3' untranslated sequence, a poly A consensus sequence and possibly a poly A run. If overlapping clones do not produce sufficient fragments to assemble a full length cDNA clone, alternative methods like RACE (PCR-based) could be used to generate the missing pieces or a full length clone.

15 c. Characterization of the human homologue full-length clone

A *NaN*-specific cDNA sequence from the full-length clone is used as a probe in Northern blot analysis to determine the messenger RNA size in human tissue for comparison with the rat and mouse messenger RNA size.

Confirmation of biological activity of the cloned cDNA is carried out using

20 methods similar to those described for the rat *NaN*.

Example 9: Polymerase chain reaction (PCR) approaches to clone the full length human *NaN* sodium channels using DNA sequence derived from rat

Total RNA and poly A<sup>+</sup> RNA is isolated from human dorsal root

25 ganglia or trigeminal ganglia or other cranial ganglia from post-mortem human material or foetuses or biopsy/surgical tissues as described above. Preparation of cDNA and PCR-based methods are then used as described previously in Example 1.

Using degenerate PCR primers derived from the rat *NaV*-specific coding sequence (see Fig. 2), the cDNA is amplified using the polymerase chain reaction [69]. A skilled artisan could utilize the many variables which can be manipulated in a PCR reaction to derive the homologous sequences required.

- 5 These include, but are not limited to, varying cycle and step temperatures, cycle and step times, number of cycles, thermostable polymerase, and  $Mg^{2+}$  concentration. A greater specificity can be achieved using nested primers derived from further conserved sequences from the *NaV* sodium channel.

- Amplification is typically performed in 60  $\mu$ l volume using 1  $\mu$ l of the  
10 first strand cDNA, 0.8 mM of each primer and 1.75 units of Expand Long Template DNA polymerase enzyme mixture (Boehringer Mannheim). Compared to conventional and thermostable DNA polymerases, Expand Long Template enzyme mixture increases the yield of the PCR products without an increase in non-specific amplification [49,50]. The PCR reaction buffer  
15 consists of 50 mM Tris-HCl (pH 9.2), 16 mM  $(NH_4)_2SO_4$ , 2.25 mM  $MgCl_2$ , 2% (v/v) DMSO and 0.1% Tween 20. As described previously [46], amplification is carried out in two stages using a programmable thermal cycler (PTC-200, MJ Research, Cambridge, MA.). First, a denaturation step at 94°C for 4 min, an annealing step at 60°C for 2 min and an elongation step at 72°C for 90 sec.  
20 Second, a denaturation step at 94°C for 1 min, an annealing step at 60°C for 1 min and an elongation step at 72°C for 90 sec. The second stage is repeated 33 times for a total of 35 cycles, with the elongation step in the last cycle extended to 10 min. In addition, control reactions are performed alongside the samples. These should be: 1) all components without cDNA, (negative control) and 2) all  
25 reaction components with primers for constitutively expressed product, *e.g.*, GAPDH.

- The products of the PCR reactions are examined on 1-1.6% (w/v) agarose gels. Bands on the gel (visualized by staining with ethidium bromide and viewing under UV light) representing amplification products of the  
30 approximate predicted size are then cut from the gel and the DNA purified.

The resulting DNA may be sequenced directly or is ligated into suitable vectors such as, but not limited to, pCR II (Invitrogen) or pGEMT (Promega). Clones are then sequenced to identify those containing sequence with similarity to the rat, mouse or partial human *NaN* sodium channel sequence.

5           Example 10: Clone analysis

Candidate clones from Example 9 are further characterized by conventional techniques. The biological activity of expression products is also confirmed using conventional techniques.

10           Example 11: Isolation of full length *NaN* sequences from human fetal tissues

Commercially available human fetal cDNA libraries and/or cDNA pools are screened with *NaN*-specific primers (by PCR) or probes (library screening) using PCR standard PCR protocols and standard library screening procedures as described above.

15           Example 12: Northern Blot of rat DRG or Trigeminal Neurons with Fragments of Rat *NaN*

20           10-30 µg total DRG and/or RNA from DRG or trigeminal (for positive tissues) and cerebral hemisphere, cerebellum and liver (for negative tissues) is electrophoresed in denaturing 1% agarose-formaldehyde gel or agarose-glyoxal gel, and then is transferred to a nylon membrane as described in achieved in multiple steps, as detailed in standard molecular biology manuals [59, 60]. Radiolabeled (specific activity of  $>10^8$  dpm/ug) or Digoxigenin-labeled RNA probes are typically used for Northern analysis. An antisense RNA probe (see Example 20, which describes *in situ* hybridization with a *NaN*-specific probe) is  
25           created by *in vitro* synthesis from a sense DNA fragment. The membrane carrying the immobilized RNA is wetted with 6x SSC, and the membrane is placed RNA-side-up in a hybridization tube. One ml formamide

- prehybridization/hybridization solution per 10 cm<sup>2</sup> of membrane is added. Prehybridization and hybridization are usually carried out in glass tubes in a commercial hybridization oven. The tubes are placed in a hybridization oven and incubated, with rotation, at 60°C for 15 min to 1 hr. The desired volume of
- 5 probe is pipetted into the hybridization tube, and the incubation is continued with rotation overnight at 60°C. The probe concentration in the hybridization solution should be 10 ng/ml if the specific activity is 10<sup>8</sup> dpm/ug or 2 ng/ml if the specific activity is 10<sup>9</sup> dpm/ug (use 2-10 ng/ml of Digoxigenin labeled probe).
- 10 The hybridization solution is poured off and an equal volume of 2x SSC/0.1% SDS is added. Incubation with rotation for 5 min at room temperature is carried out. The wash solution is changed, and this step is repeated. To reduce background, it may be beneficial to double the volume of the wash solutions. The wash solution is replaced with an equal volume of 0.2x
- 15 SSC/0.1% SDS and the tube is incubated for 5 min with rotation at room temperature. The wash solution is changed and this step is repeated (this is a low-stringency wash). For moderate or high stringency conditions, further washes are done with wash solutions pre-warmed to moderate (42°C) or high (68°C) temperatures. The final wash solution is removed and the membrane
- 20 rinsed in 2x SSC at room temperature. Autoradiography is then performed for up to 1 week. Alternatively, signal is detected using chemiluminescence technology (Amersham) if non-radioactive probes are used. The transcript size is calculated from the signal from the gel in comparison with gel molecular weight standard markers.
- 25 **Example 13: Tissue specific distribution of NaN by RT-PCR**
- NaN*-specific forward (5' CCCTGCTGCGCTCGGTGAAGAA 3') and reverse primer (5' GACAAAGTAGATCCCAGAGG 3'), were used in RT-PCR assays using cDNA template prepared from multiple rat. These primers amplify *NaN* sequence between nucleotides 765 and 1156 (392 bp) and are *NaN*-specific



as judged by lack of similarity to sequences in the database (using programs like BLASTN from the National Library of Medicine). Amplification was typically performed in a 60  $\mu$ l volume using 1  $\mu$ l of the first strand of cDNA, 0.8  $\mu$ M of each primer and 1.75 units of Expand Long Template DNA polymerase enzyme mixture (Boehringer Mannheim). Compared to conventional and thermostable DNA polymerases, Expand Long Template enzyme mixture increases the yield of the PCR products without an increase in non-specific amplification [49, 50]. The PCR reaction buffer consisted of 50 mM Tris-HCl (pH 9.2), 16mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.25 mM MgCl<sub>2</sub>, 2% (v/v) DMSO and 0.1% Tween 20. As described previously [71], amplification was carried out in two stages using a programmable thermal cycler (PTC-200, MJ Research, Cambridge, MA.). First, a denaturation step is performed at 94°C for 4 min., followed by an annealing step at 60°C for 2 min, and then an elongation step at 72°C for 90 sec. Second, a denaturation step is performed at 94°C for 1 min, followed by an annealing step at 60°C for 1 min, and then an elongation step at 72°C for 90 sec. The second stage was repeated 33 times for a total of 25-35 cycles, with the elongation step in the last cycle extended to 10 min.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to ensure that a lack of *NaN* signals in different tissues was not due to degraded templates or presence of PCR inhibitors. Rat GAPDH sequences were co-amplified using primers which amplify a 66 bp product that corresponds to nucleotides 328-994 (accession number: M17701). The amplified product spans multiple exon/intron splice sites, based on the structure of the human gene [72]. Dnase I treatment was routinely performed prior to reverse transcription to prevent amplification of GAPDH sequences from genomic processed pseudogenes that may have contaminated the total RNA preparation [73].

*NaN* is primarily and preferentially expressed in DRG and trigeminal ganglia neurons. Figure 4 shows the results of screening by RT-PCR for the expression of *NaN* in various neuronal and non-neuronal tissues. Lanes 1, 2, 4,

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9 and 16 show a single amplification product co-migrating with the 400 bp marker, consistent with *NaN*-specific product of 392 bp. Lanes 1 and 16, 2, 4 and 9 contain products using DRG, cerebral hemisphere, retina, and trigeminal ganglia, respectively. Using this assay, *NaN* was not detected in cerebellum, 5 optic nerve, spinal cord, sciatic nerve, superior cervical ganglia, skeletal muscle, cardiac muscle, adrenal gland, uterus, liver or kidney (lanes 3, 5-8, and 10-15, respectively). The attenuated *NaN* signal in cerebral hemisphere and retina, and the absence of this signal in the remaining tissues is not due to degraded RNA or the presence of PCR inhibitors in the cDNA templates as comparable 10 GAPDH amplification products were obtained in a parallel set of PCR reaction (data not shown).

Example 14: Transformation of a Host Cell with the *NaN* Coding Sequence

Transformed host cells for the measurement of  $\text{Na}^+$  current or 15 intracellular  $\text{Na}^+$  levels are usually prepared by co-transfecting constructs into cells such as HEK293 cells with a fluorescent reporter plasmid (pGreen Lantern-1, Life Technologies, Inc.) using the calcium-phosphate precipitation technique [27]. HEK293 cells are typically grown in high glucose DMEM (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, 20 Inc). After 48 hrs, cells with green fluorescence are selected for recording [28].

For preparation of cell lines continuously expressing recombinant channels, the *NaN* construct is cloned into other vectors that carry a selectable marker in mammalian cells. Transfections are carried out using the calcium phosphate precipitation technique [27]. Human embryonic kidney (HEK-293), 25 chinese-hamster ovary (CHO) cells, or other suitable cell-lines are grown under standard tissue culture conditions in Dulbeccos's modified Eagle's medium supplemented with 10% fetal bovine serum. The calcium phosphate-DNA mixture is added to the cell culture medium and left for 15-20 hr, after which time the cells are washed with fresh medium. After 48 hrs, antibiotic (G418,

Geneticin, Life Technologies) is added to select for cells which have acquired neomycin resistance. After 2-3 weeks in G418, 10-20 isolated cell colonies are harvested using sterile 10ml pipette tips. Colonies are grown for another 4-7 days, split and subsequently tested for channel expression using whole-cell  
5 patch-clamp recording techniques and RT-PCR.

Example 15: Production of NaN specific Antibodies

Antibodies specific to the rat, mouse or human NaN are produced with an immunogenic NaN-specific peptide by raising polyclonal antibodies in rabbits. In one example, the peptide **CGPNPASNKDCFEKEKDS**ED (rat  
10 amino acids 285-304) was selected because it fits the criteria for immunogenicity and surface accessibility. This peptide sequence does not match any peptide in the public databases. The underlined cysteine (C) residue was changed to Alanine (A) to prevent disulfide bond formation. This amino acid change is not expected to significantly affect the specificity of the  
15 antibodies.

Peptide synthesis, rabbit immunization, and affinity purification of the anti-peptide antibodies were performed using standard procedures. Purified antibodies were then tested on DRG neurons in culture. Immunostaining procedures using these antibodies before and after blocking with excess peptide  
20 were performed according to standard procedures.

DRG neurons after 16-24 h in culture were processed for immunocytochemical detection of NaN protein as follows. Coverslips were washed with complete saline solution (137 mM NaCl, 5.3 mM KCl, 1 mM M902 25 mM sorbitol, 10 mM HEPES, 3 mM CaCl<sub>2</sub> pH 7.2), fixed with 4%  
25 paraformaldehyde in 0.14 M phosphate buffer for 10 min at 4°C, washed with three 5-min with phosphate-buffered saline (PBS), and blocked with PBS containing 20% normal goat serum, 1% bovine serum albumin and 0.1% Triton X-100 for 15 minutes. The coverslips were incubated in anti-NaN antibody (1:100 dilution) at 4°C overnight. Following overnight incubation,

coverslips were washed extensively in PBS and then incubated with goat anti-rabbit IgG-conjugated to Cy3 (1:3000; Amersham) for 2 h at room temperature. The coverslips were rinsed with PBS and mounted onto glass slides with Aqua-poly-mount. The neurons were examined with a Leitz Aristoplan light  
5 microscope equipped with epifluorescence and images were captured with a Dage DC330T color camera and Scion CG-7 color PCI frame grabber (see Figure 7).

Example 16: NaN expression is altered in a neuropathic pain model

The CCI model of neuropathic pain (Bennett and Xie) was used to study  
10 the plasticity of sodium channel expression in DRG neurons. Twenty two adult, femal Sprague-Dawley rats, weighing 240-260g, were anesthetized with pentobarbital sodium (50 mg/kg ip) and the right sciatic nerve exposed at the mid-thigh. Four chromic gut (4-0) ligatures were tied loosely around the nerve as described by Bennett and Xie (1988) *Pain* 33, 87-107. The incision site was  
15 closed in layers and a bacteriostatic agent administered intramuscularly.

Previous studies have shown that transection of the sciatic nerve induces dramatic changes in sodium currents of axotomized DRG neurons, which is paralleled by significant changes to transcripts of various sodium channels expressed in these neurons. Sodium currents that are TTX-R and the transcripts  
20 of two TTX-R sodium channels (SNS/PN3 and NaN) are significantly attenuated while a rapidly repriming silent TTX-S current emerges and the transcript of  $\alpha$ -III sodium channel, which produces a TTX-S current, is up-regulated. We have discovered that CCI-induced changes in DRG neurons, 14 days post-surgery, mirror those of axotomy. Transcripts of NaN and SNS, the  
25 two sensory neuron-specific TTX-R channels, are significantly down-regulated as is the TTX-R sodium current, while transcripts of the TTX-S  $\alpha$ -III sodium channel are up-regulated, in small diameter DRG neurons. These changes may be partly responsible for making DRG neurons hyperexcitable, that contributes to the hyperalgesia that results from this injury.

Example 17: Assays for agents which modulate the activity of the NaN channel using patch clamp methods

Cells lines expressing the cloned Na<sup>+</sup> channel are used to assay for agents which modulate the activity of the NaN channel, *e.g.*, agents which block  
5 or inhibit the channel or enhance channel opening. Since the channel activation is voltage dependent, depolarizing conditions may be used for observation of baseline activity that is modified by the agent to be tested. Depolarization may be achieved by any means available, for example, by raising the extracellular potassium ion concentration to about 20 to 40 nM, or by repeated electrical  
10 pulses.

The agent to be tested is incubated with HEK 293 or other transformed cells that express the Na<sup>+</sup> channel [28]. After incubation for a sufficient period of time, the agent induced changes in Na<sup>+</sup> channel activity can be measured by patch-clamp methods [29]. Data for these measurements are acquired on a  
15 MacIntosh Quadra 950, or similar computer, using a program such as Pulse (v 7.52, HEKA, German). Fire-polished electrodes (0.8-1.5 MW) are fabricated from capillary glass using a Sutter P-87 puller or a similar instrument. Cells are usually only considered for analysis if initial seal resistance is <5 Gohm, they have high leakage currents (holding current <0.1 nA at -80 mV), membrane  
20 blebs, and an access resistance <5 Mohm. Access resistance is monitored and data is not used if resistance changes occur. Voltage errors are minimized using series resistance compensation and the capacitance artifact will be canceled as necessary using computer-controlled amplifier circuitry or other similar methods.

25 For comparisons of the voltage dependence of activation and inactivation, cells with a maximum voltage error of <10 mV after compensation are usually used. Linear leak subtraction is used for voltage clamp recordings. Membrane currents are typically filtered at 5 KHz and sampled at 20 KHz. The pipette solution contains a standard solution such as: 140 mM CsF, 2 mM  
30 MgCl<sub>2</sub>, 1 mM EGTA, and 10 mM Na-HEPES (pH 7.3). The standard bathing

solution is a standard solution such as 140 mM NaCl, 3 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10 mM HEPES, and 10 mM glucose (pH 7.3).

Tetrodotoxin (TTX)-resistant and TTX-sensitive Na<sup>+</sup> currents are measured by exposure to appropriate concentrations of TTX and/or by pre-pulse protocols which distinguish between TTX-sensitive and TTX-resistant currents on the basis of their distinct steady-state inactivation properties [22,55].

Data are collected using standard pulse protocols and are analyzed to measure sodium current properties that include voltage-dependence, steady-state characteristics, kinetics, and re-priming. Measurements of current amplitude and cell capacitance provides an estimate of Na<sup>+</sup> current density, thereby permitting comparisons of channel density under different conditions [22,30]. Cells are studied in the current clamp mode to study patterns of spontaneous and evoked action potential generation, threshold for firing, frequency response characteristics, and response to de- and hyperpolarization, and other aspects of electrogenesis [55]. These measurements are carried out both in control cells expressing *NaV* and in cells expressing *NaV* that also have been exposed to the agent to be tested.

Example 18: Assays for agents which modulate the activity of the NaN channel by the measurement of Intracellular Sodium [Na<sup>+</sup>]

The agent to be tested is incubated with cells exhibiting NaN channel activity. After incubation for a sufficient period of time, the agent induced changes in Na<sup>+</sup> channel are measured by ratiometric imaging of [Na<sup>+</sup>], using SBFI. In this method, cytosolic-free Na<sup>+</sup> is measured using an indicator for Na<sup>+</sup>, such as SBFI (sodium-binding benzofuran isophthalate; [33]) or a similar dye. Cells are first loaded with the membrane-permeable acetoxymethyl ester form of SBFI (SBFI/AM) or a similar dye (usually dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM). Recordings are obtained on the stage of a microscope using a commercially available ratiometric imaging setup (e.g., from Georgia Instruments). Excitation light is provided at appropriate

wavelengths (*e.g.*, 340:385 nm). Excitation light is passed to the cells through a dichroic reflector (400 nm) and emitted light above 450 nm was collected.

Fluorescence signals are amplified, *e.g.*, by an image intensifier (GenIISyS) and collected with a CCD camera, or similar device, interfaced to a frame grabber.

- 5 To account for fluorescence rundown, the fluorescence ratio 340:385 is used to assay cytosolic-free  $\text{Na}^+$ .

- For calibration of SBFI's fluorescence, cells are perfused with calibration solutions containing known  $\text{Na}^+$  concentrations (typically 0 and 30 mM, or 0, 30, and 50 mM  $[\text{Na}^+]$ , and gramicidin and monensin. As reported by
- 10 Rose and Ransom [34], the 345/390 nm fluorescence ratio of intracellular SBFI changes monotonically with changes in  $[\text{Na}^+]_i$ . Experiments are repeated on multiple (typically at least 4) different coverslips, providing statistically significant measurements of intracellular sodium in control cells, and in cells exposed to various concentrations of agents that may block, inhibit or enhance
- 15 the activity of the channel. Use of this method is illustrated in Sontheimer *et al.* [32].

Example 19: Assays for agents which modulate the activity of the  $\text{Na}^+$  channel by scintigraphic imaging

- Cells lines expressing the cloned  $\text{Na}^+$  channel are used to assay for
- 20 agents which modulate the activity of the  $\text{Na}^+$  channel, *e.g.*, agents which block the channel or enhance channel opening. For example, the agent to be tested is incubated with HEK 293 or other transformed cells that express the  $\text{Na}^+$  channel [28]. After incubation for a sufficient period of time, the agent induced changes in  $\text{Na}^+$  channel activity are detected by the measurement of  $\text{Na}^+$  influx by
- 25 isotopic methods.  $^{22}\text{Na}$  is a gamma emitter and can be used to measure  $\text{Na}^+$  flux [31] and  $^{86}\text{Rb}^+$  can be used to measure  $\text{Na}^+/\text{K}^+$  ATPase activity which provides a measure of  $\text{Na}^+$  channel activity [32]  $^{86}\text{Rb}^+$  ions are taken up by the  $\text{Na}^+/\text{K}^+$  ATPase like  $\text{K}^+$  ions, but have the advantage of a much longer half-life than  $^{42}\text{K}^+$  [35]. Thus, measurement of the unidirectional ouabain-sensitive  $^{86}\text{Rb}^+$

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uptake provides a quantitative method for assaying  $\text{Na}^+/\text{K}^+$ -ATPase activity which follows action potentials.

Following incubation of cell expressing *NaV* to the isotope, the cellular content of the isotope is measured by liquid scintillation counting or a similar method, and cell protein is determined using a method such as the bicinchoninic acid protein assay [36] following the modifications [37] for cultured cells.  $^{22}\text{Na}$  and  $^{86}\text{Rb}^+$  fluxes are determined in the presence and absence of agents that may block, inhibit, or enhance  $\text{Na}^+$ . This permits determination of the actions of these agents on *NaV*.

10      Example 20: In situ hybridization

          a. Probes

          Probes are prepared as described above in Example 5.

          b. DRG Neuron Culture

          Cultures of DRG neurons from adult rats were established as described previously [70]. Briefly, lumbar ganglia (L4, L5) from adult Sprague Dawley female rats were freed from their connective sheaths and incubated sequentially in enzyme solutions containing collagenase and then papain. The tissue was triturated in culture medium containing 1:1 Dulbecco's modified Eagle's medium (DMEM) and Hank's F12 medium and 10% fetal calf serum, 1.5 mg/ml trypsin inhibitor, 1.5 mg/ml bovine serum albumin, 100 U/ml penicillin and 0.1 mg/ml streptomycin and plated at a density of 500-1000 cells/mm<sup>2</sup> on polyornithine/laminin coated coverslips. The cells were maintained at 37°C in a humidified 95% air/5% CO<sub>2</sub> incubator overnight and then processed for *in situ* hybridization cytochemistry as described previously [56, 57]. Trigeminal ganglia can be cultured by a skilled artisan using similar methods.

          c. Tissue Preparation

          Adult female Sprague Dawley rats were deeply anesthetized, *e.g.*, with chloral hydrate and perfused through the heart, first with a phosphate-buffered



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saline (PBS) solution and then with a 4% paraformaldehyde in 0.14 M Sorensen's phosphate buffer, pH 7.4, at 4°C. Following perfusion fixation, dorsal root ganglia at levels L4 and L5 and trigeminal ganglia were collected and placed in fresh fixative at 4°C. After 2-4 hours, the tissue was transferred  
5 to a solution containing 4% paraformaldehyde and 30% sucrose in 0.14 M phosphate buffer and stored overnight at 4°C. Fifteen µm sections were cut and placed on poly-L-lysine-coated slides. The slides were processed for *in situ* hybridization cytochemistry as previously described [24, 56]. Following *in situ* hybridization cytochemistry, the slides were dehydrated, cleared and mounted  
10 with Permount. The results are shown in Fig. 5.

Sections of DRG hybridized with *NaV* sense riboprobe showed no specific labeling (panel C, Fig. 5). In DRG (panel A, Fig. 5) and trigeminal (panel B) sections hybridized with a *NaV* antisense riboprobe, with the *NaV* signal present in most small (<30 mm diam.) neurons; in contrast, most large  
15 (>30 mm diam.) neurons did not exhibit *NaV* hybridization signal. Sections of spinal cord, cerebellum and liver hybridized with an antisense *NaV* riboprobe showed no specific signal (panels D, E and F respectively).

#### Example 21: Microsatellite Sequences

The following are the murine intronic microsatellite sequences. These  
20 microsatellites may be polymorphic in the human SCN11a gene and could be used as markers to screen for mutant alleles that are associated with a disease. Such screening methods, for instance, hybridization or amplification assays, are readily available. See Sambrook *et al.* or Ausubel *et al.*





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**Intron 12; microsatellite is dpydG (dt/dCdG)**

GTGCTGGGATCAAAGGCGTGCGCCGCCACCGCCCGGCCCC  
TTTTTATGTTTCAAATTTACTTTATCATGTGCACGTGTGTGGGTGCG  
TGCAATGTGTGTGCGTGCGTGTCGTGTGNGTGTGNGTGTGTGTGTGT  
5 GTGTGTGTGTGTGTGTGTGTGTG

**Intron 14; microsatellite is dCdA**

CACACACACACACACACACACACACACACACACACA  
CACACACACACACTTGCATCTTTGAGTTAATTGGATAGGCTGAGT  
CTTACACCGGAATCATACTGTTGC

10            Intron 15A; microsatellite is dCdA

CCAATGAGAGACTCTTGTCTCAAAAAAGCCATGGTGTCCAGA  
TCCTGAGGAATAACACCTAAGAATGTGCTCTGGCCTGAAAACACAC  
ACACACACACACACACACACACACACACAGTTTTATTTATTTA  
TTTAAAAAATATGTCTCTAGGCATTGCTGAAATGTCTCCTACAGGA  
15 TTAAGTCAACCAGAGCCA

It should be understood that the foregoing discussion and examples merely present a detailed description of certain preferred embodiments. It will be apparent to those of ordinary skill in the art that various modification and equivalents can be made without departing from the spirit and scope of the invention.

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**CLAIMS**

1. An isolated nucleic acid molecule selected from the group consisting of a nucleic acid molecule comprising the sequence shown in Fig. 1, Fig. 7A or Fig. 8A, a nucleic acid molecule which encodes the amino acid sequence of Figure 2, Figure 7B, Figure 8B or allelic variants of said sequences, and a nucleic acid molecule that hybridizes to one of the foregoing sequences under stringent conditions.
2. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes a voltage gated Na<sup>+</sup> channel that is preferentially expressed in dorsal root ganglia or trigeminal ganglia.
3. The isolated nucleic acid of claim 2, wherein the nucleic acid encodes the human NaN sodium channel.
4. An expression vector comprising the isolated nucleic acid of any of claims 1 to 3, alone or together with appropriate regulatory and expression control elements.
5. A host cell transformed with the expression vector of claim 4.
6. A Na<sup>+</sup> channel encoded by the isolated nucleic acid of any of claims 1 to 3.
7. The Na<sup>+</sup> channel of claim 6, having the amino acid sequence of Figure 2.
8. A protein molecule having the amino acid sequence of Fig. 2, Figure 7B, Figure 8B or a peptide fragment thereof.

9. A protein encoded by the isolated nucleic acid of claim 6.

10. A method to identify an agent that modulates the activity of the Na<sup>+</sup> channel of claim 6, comprising the steps of bringing the agent into contact with a cell that expresses the Na<sup>+</sup> channel on its surface and measuring any resultant changes in the sodium current, resultant change in membrane potential or change in intracellular Na<sup>+</sup>.

11. The method of claim 10, wherein the measuring step is accomplished with voltage clamp measurements or measurement of membrane potential.

12. The method of claim 10, wherein the measuring step is accomplished by measuring the level of intracellular sodium.

13. The method of claim 10, wherein the measuring step is accomplished by measuring sodium influx.

14. The method of claim 13, wherein the sodium influx is measured using <sup>22</sup>Na or <sup>86</sup>Rb.

15. A method to identify an agent that modulates the transcription or translation of mRNA encoding the Na<sup>+</sup> channel of claim 6, comprising the steps of bringing the agent into contact with a cell that expresses the Na<sup>+</sup> channel on its surface and measuring the resultant level of expression of the Na<sup>+</sup> channel.

16. A method to treat pain, paraesthesia and/or hyperexcitability phenomena in an animal or human subject by administering an effective amount

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of an agent capable of altering  $\text{Na}^+$  current flow through NaN channels in DRG or trigeminal neurons.

17. A method to treat pain, paraesthesia and/or hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of modulating the transcription or translation of mRNA encoding the  $\text{Na}^+$  channel of claim 6.

18. An isolated nucleic acid that is antisense to the nucleic acid of claim 1 and of sufficient length to modulate the expression of NaN channel in a cell containing the mRNA.

19. A scintigraphic method to image the loci of pain generation or provide a measure of the level of pain associated with DRG or trigeminal neuron mediated hyperexcitability in an animal or human subject by administering labeled monoclonal antibodies or other labeled ligands specific for the NaN  $\text{Na}^+$  channel.

20. A method to identify tissues, cells and cell types that express the NaN sodium channel, comprising the step of detecting NaN on the cell surface or intracellularly.

21. A method to identify tissues, cells and cell types that express NaN comprising the step of detecting the presence therein of NaN encoding mRNA.

22. A method of producing a transformed cell that expresses an exogenous NaN encoding nucleic acid, comprising the step of transforming the cell with an expression vector comprising the isolated nucleic acid of any of

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claims 1 to 3, together with appropriate regulatory and expression control elements.

23. An isolated antibody specific for the *NaN* channel or polypeptide fragment thereof.

24. The isolated antibody of claim 23, wherein the antibody is labeled.

25. A method of producing recombinant NaN protein, comprising the step of culturing the transformed host of claim 5 under conditions in which the NaN sodium channel or protein is expressed, and recovering the NaN protein.

26. A therapeutic composition comprising an effective amount of an agent capable of altering, such as by increasing or decreasing, the rapidly repriming current flow in axotomized, inflamed or otherwise injured DRG neurons.

27. A method to treat acute pain or acute or chronic neuropathic or inflammatory pain and hyperexcitability phenomena in an animal or a human patient by administering the therapeutic composition of claim 26.

28. A method to screen candidate compounds for use in treating pain and hyperexcitability phenomena by testing their ability to upregulate or downregulate the NaN channel mRNA in axotomized, inflamed or otherwise injured DRG neurons.

29. A method of screening for a mutant NaN allele in a nucleic acid sample comprising the step of hybridizing an intronic microsatellite sequence of Example 20 to the sample under conditions of sufficient stringency to produce a clear signal.

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**FIG. 1A**

Nucleotide sequence of rat NaN. Translation initiation begins  
at position 41 (ATG). Reading frame ends at position 5336 (TGA).

```

1  ACGGTGCCCT GATCCTCTGT ACCAGGAAGA CAGGGTGAAG ATGGAGGAGA
51  GGTACTACCC GGTGATCTTC CCGGACGAGC GGAATTTCCTG CCCCTTCACT
101 TCCGACTCTC TGGCTGCCAT AGAGAAGCGG ATTGCTATCC AAAAGGAGAG
151 GAAGAAGTCC AAAGACAAGG CGGCAGCTGA GCCCCAGCCT CGGCCTCAGC
201 TTGACCTAAA GGCTCCAGG AAGTTACCTA AGCTTTATGG TGACATTCCC
251 CCTGAGCTTG TAGCGAAGCC TCTGGAAGAC CTGGACCCAT TCTACAAAGA
301 CCATAAGACA TTCATGGTGT TGAACAAGAA GAGAACAATT TATCGCTTCA
351 GCGCCAAGCG GGCTTGTTTC ATTCTGGGGC CTTTTAATCC CCTCAGAAGC
401 TTAATGATTC GTATCTCTGT CCATTCAGTC TTTAGCATGT TCATCATCTG
451 CACGGTGATC ATCAACTGTA TGTTTCATGGC GAATTCTATG GAGAGAAGTT
501 TCGACAACGA CATTCCCGAA TACGTCTTCA TTGGGATTTA TATTTTAGAA
551 GCTGTGATTA AAATATTGGC AAGAGGCTTC ATTGTGGATG AGTTTTCCTT
601 CCTCCGAGAT CCGTGGAAC TGGCTGGACTT CATGTGCATT GGAACAGCGA
651 TCGCAACTTG TTTTCCGGGC AGCCAAGTCA ATCTTTCAGC TCTTCGTACC
701 TTCCGAGTGT TCAGAGCTCT GAAGGCGATT TCAGTTATCT CAGGTCGTAA
751 GGTCAATCGT GGTGCCCTGC TGCGCTCGGT GAAGAAGCTG GTAGACGTGA
801 TGGTCCTCAC TCTCTTCTGC CTCAGCATCT TTGCCCTGGT CGGTCAGCAG
851 CTGTTCATGG GAATTCTGAA CCAGAAGTGT ATTAAGCACA ACTGTGGCCC
901 CAACCCTGCA TCCAACAAGG ATTGTTTTGA AAAGGAAAAA GATAGCGAAG
951 ACTTCATAAT GTGTGGTACC TGGCTCGGCA GCAGACCCTG TCCCAATGGT
1001 TCTACGTGCG ATAAAACCAC ATTGAACCCA GACAATAATT ATACAAAGTT
1051 TGACAACTTT GGCTGGTCCT TTCTCGCCAT GTTCCGGGTT ATGACTCAAG
1101 ACTCCTGGGA GAGGCTTTAC CGACAGATCC TGCGGACCTC TGGGATCTAC
1151 TTTGTCTTCT TCTTCGTGGT GGTCATCTTC CTGGGCTCCT TCTACCTGCT
1201 TAACCTAACC CTGGCTGTTG TCACCATGGC TTATGAAGAA CAGAACAGAA
1251 ATGTAGCTGC TGAGACAGAG GCCAAGGAGA AAATGTTTCA GGAAGCCCAG
1301 CAGCTGTTAA GGGAGGAGAA GGAGGCTCTG GTTGCCATGG GAATTGACAG
1351 AAGTTCCCTT AATTCCTTTC AAGCTTCATC CTTTCCCCCG AAGAAGAGGA
1401 AGTTTTTCGG TAGTAAGACA AGAAAGTCCT TCTTTATGAG AGGGTCCAAG
1451 ACGGCCAAG CCTCAGCGTC TGATTTCAGAG GACGATGCCT CTAAAAATCC
1501 ACAGCTCCTT GAGCAGACCA AACGACTGTC CCAGAACTTG CCAGTGATC
1551 TCTTTGATGA GCACGTGGAC CCCCTCCACA GGCAGAGAGC GCTGAGCGCT
1601 GTCAGTATCT TAACCATCAC CATGCAGGAA CAAGAAAAAT TCCAGGAGCC
1651 TTGTTTCCCA TGTGGGAAAA ATTTGGCCTC TAAGTACCTG GTGTGGGACT

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**FIG. 1B**

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1701 GTAGCCCTCA ATGGCTGTGC ATAAAGAAGG TCCTGCGGAC CATCATGACG  
1751 GATCCCTTTA CTGAGCTGGC CATCACCATC TGCATCATCA TCAATACCGT  
1801 TTTCTTAGCC GTGGAGCACC ACAACATGGA TGACAACTTA AAGACCATAC  
1851 TGAAAATAGG AAACCTGGGT TTCACGGGAA TTTTCATAGC GGAAATGTGT  
1901 CTCAAGATCA TCGCGCTCGA CCCTTACCAC TACTTCCGGC ACGGCTGGAA  
1951 TGTTTTTGAC AGCATCGTGG CCCTCCTGAG TCTCGCTGAT GTGCTNTACA  
2001 ACACACTGTC TGATAACAAT AGGTCTTTCT TGGCTTCCCT CAGAGTGCTG  
2051 AGGGTCTTCA AGTTAGCCAA ATCCTGGCCC ACGTTAAACA CTCTCATTA  
2101 GATCATCGGC CACTCCGTGG GCGCGCTTGG AAACCTGACT GTGGTCTCTGA  
2151 CTATCGTGGT CTTCATCTTT TCTGTGGTGG GCATGCGGCT CTTGCGCACC  
2201 AAGTTTAAAC AGACCGCCTA CGCCACCCAG GAGCGGCCCA GGCGGCGCTG  
2251 GCACATGGAT AATTTCTACC ACTCCTTCCT GGTGGTGTTC CGCATCCTCT  
2301 GTGGGGAATG GATCGAGAAC ATGTGGGGCT GCATGCAGGA TATGGACGGC  
2351 TCCCCGTTGT GCATCATTGT CTTTGTCTG ATAATGGTGA TCGGGAAGCT  
2401 TGTGGTGCTT AACCTCTTCA TTGCCTTGCT GCTCAATTCC TTCAGCAATG  
2451 AGGAGAAGGA TGGGAGCCTG GAAGGAGAGA CCAGGAAAAC CAAAGTGCAG  
2501 CTAGCCCTGG ATCGGTTCCG CCGGGCCTTC TCCTTCATGC TGCACGCTCT  
2551 TCAGAGTTTT TGTGCAAGA AATGCAGGAG GAAAACTCG CCAAAGCCAA  
2601 AAGAGACAAC AGAAAGCTTT GCTGGTGAGA ATAAAGACTC AATCCTCCCC  
2651 GATGCGAGGC CCTGGAAGGA GTATGATACA GACATGGCTT TGTACACTGG  
2701 ACAGGCCGGG GCTCCGCTGG CCCCCTCGC AGAGGTAGAG GACGATGTGG  
2751 AATATTGTGG TGAAGGCGGT GCCCTACCCA CCTCACAACA TAGTGCTGGA  
2801 GTTCAGGCCG GTGACCTCCC TCCAGAGACC AAGCAGCTCA CTAGCCCCGA  
2851 TGACCAAGGG GTTGAAATGG AAGTATTTTC TGAAGAAGAT CTGCATTTAA  
2901 GCATACAGAG TCCTCGAAAG AAGTCTGACG CAGTGAGCAT GCTCTCGGAA  
2951 TGCAGCACAA TTGACCTGAA TGATATCTTT AGAAATTTAC AGAAAACAGT  
3001 TTCCCCCAA AAGCAGCCAG ATAGATGCTT TCCCAAGGGC CTTAGTTGTC  
3051 ACTTTCTATG CCACAAAACA GACAAGAGAA AGTCCCCCTG GGTCTGTGG  
3101 TGGAACATTC GGAAAACCTG CTACCAAATC GTGAAGCACA GCTGGTTTGA  
3151 GAGTTTCATA ATCTTTGTTA TTCTGCTGAG CAGTGGAGCG CTGATATTTG  
3201 AAGATGTCAA TCTCCCCAGC CGGCCCAAG TTGAGAAATT ACTAAGGTGT  
3251 ACCGATAATA TTTTCACATT TATTTTCCTC CTGGAAATGA TCCTGAAGTG  
3301 GGTGGCCTTT GGATTCCGGA GGTATTTTAC CAGTGCCTGG TGCTGGCTTG  
3351 ATTTCTCAT TGTGGTGGTG TCTGTGCTCA GTCTCATGAA TCTACCAAGC  
3401 TTGAAGTCCT TCCGGACTCT GCGGGCCCTG AGACCTCTGC GGGCGCTGTC  
3451 CCAGTTTGAA GGAATGAAGG TTGTCGTCTA CGCCCTGATC AGCGCCATAC  
3501 CTGCCATTCT CAATGTCTTG CTGGTCTGCC TCATTTTCTG GCTCGTATTT  
3551 TGTATCTTGG GAGTAAATTT ATTTTCTGGG AAGTTTGGA GGTGCATTAA  
3601 CGGGACAGAC ATAAATATGT ATTTGGATTT TACCGAAGTT CCGAACCAGAA

**FIG. 1C****3/21**

3651 GCCAATGTAA CATTAGTAAT TACTCGTGGA AGGTCCCGCA GGTCAACTTT  
 3701 GACAACGTGG GGAATGCCTA TCTCGCCCTG CTGCAAGTGG CAACCTATAA  
 3751 GGGCTGGCTG GAAATCATGA ATGCTGCTGT CGATTCCAGA GAGAAAGACG  
 3801 AGCAGCCGGA CTTTGAGGCG AACCTCTACG CGTATCTCTA CTTTGTGGTT  
 3851 TTTATCATCT TCGGCTCCTT CTTTACCCTG AACCTCTTTA TCGGTGTTAT  
 3901 TATTGACAAC TTCAATCAGC AGCAGAAAAA GTTAGGTGGC CAAGACATTT  
 3951 TTATGACAGA AGAACAGAAG AAATATTACA ATGCAATGAA AAAGTTAGGA  
 4001 ACCAAGAAAC CTCAAAAGCC CATCCCAAGG CCCCTGAACA ANTGTCAAGC  
 4051 CTTTGTGTTC GACCTGGTCA CAAGCCATGT CTTTGACGTC ATCATTCTGG  
 4101 GTCTTATTGT CTTAAATATG ATTATCATGA TGGCTGAATC TGCCGACCAG  
 4151 CCCAAAGATG TGAAGAAAAC CTTTGATATC CTCAACATAG CCTTCGTGGT  
 4201 CATCTTTACC ATAGAGTGTC TCATCAAAGT CTTTGCTTTG AGGCAACACT  
 4251 ACTTCACCAA TGGCTGGAAC TTATTTGATT GTGTGGTCGT GGTTCTTTCT  
 4301 ATCATTTAGTA CCCTGGTTTC CCGCTTGGAG GACAGTGACA TTTCTTTCCC  
 4351 GCCCACGCTC TTCAGAGTCG TCCGCTTGGC TCGGATTGGT CGAATCCTCA  
 4401 GGCTGGTCCG GGCTGCCCCG GGAATCAGGA CCCTCCTCTT TGCTTTGATG  
 4451 ATGTCTCTCC CCTCTCTCTT CAACATCGGT CTGCTGCTCT TCCTGGTGAT  
 4501 GTTCATTTAC GCCATCTTTG GGATGAGCTG GTTTTCCAAA GTGAAGAAGG  
 4551 GCTCCGGGAT CGACGACATC TTCAACTTCG AGACCTTTAC GGGCAGCATG  
 4601 CTGTGCCTCT TCCAGATAAC CACTTCGGCT GGCTGGGATA CCCTCCTCAA  
 4651 CCCCATGCTG GAGGCAAAAG AACACTGCAA CTCCTCCTCC CAAGACAGCT  
 4701 GTCAGCAGCC GCAGATAGCC GTCGTCTACT TCGTCAGTTA CATCATCATC  
 4751 TCCTTCCTCA TCGTGGTCAA CATGTACATC GCTGTGATCC TCGAGAACTT  
 4801 CAACACAGCC ACGGAGGAGA GCGAGGACCC TCTGGGAGAG GACGACTTTG  
 4851 AAATCTTCTA TGAGGTCTGG GAGAAGTTTG ACCCCGAGGC GTCGCAGTTC  
 4901 ATCCAGTATT CGGCCCTCTC TGACTTTGCG GACGCCCTGC CGGAGCCGTT  
 4951 GCGTGTGGCC AAGCCGAATA AGTTTCAGTT TCTAGTGATG GACTTGCCCCA  
 5001 TGGTGATGGG CGACCGCCTC CATTGCATGG ATGTTCTCTT TGCTTTCACT  
 5051 ACCAGGGTCC TCGGGGACTC CAGCGGCTTG GATACCATGA AAACCATGAT  
 5101 GGAGGAGAAG TTTATGGAGG CCAACCCCTT TAAGAAGCTC TACGAGCCCA  
 5151 TAGTCACCAC CACCAAGAGG AAGGAGGAGG AGCAAGGCGC CGCCGTCATC  
 5201 CAGAGGGCCT ACCGGAACA CATGGAGAAG ATGGTCAAAC TGAGGCTGAA  
 5251 GGACAGGTCA AGTTCATCGC ACCAGGTGTT TTGCAATGGA GACTTGTCCTA  
 5301 GCTTGGATGT GGCCAAGGTC AAGGTTTACA ATGACTGAAC CCTCATCTCC  
 5351 ACCCCTACCT CACTGCCTCA CAGCTTAGCC TCCAGCCTCT GGCGAGCAGG  
 5401 CGGCAGACTC ACTGAACACA GGCCGTTCTGA TCTGTGTTTT TGGCTGAACG  
 5451 AGGTGACAGG TTGGCGTCCA TTTTTAAATG ACTCTTGGA AGATTTTCATG  
 5501 TAGAGAGATG TTAGAAGGGA CTGCAAAGGA CACCGACCAT AACGGAAGGC



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***FIG. 1D***

5601 CCAGGAAAAC TTCAGATTGT GTTCTCAGTA CATTCCCCAA TGTGTCTGTT  
5651 CGGTGTTTTG AGTATGTGAC CTGCCACATG TAGCTCTTTT TTGCATGTAC  
5701 GTCAAAACCC TGCAGTAAGT TAATAGCTTG CTACGGGTGT TCCTACCAGC  
5751 ATCACAGAAT TGGGTGTATG ACTCAAACCT AAAAGCATGA CTCTGACTTG  
5801 TCAGTCAGCA CCCCAGCTTT CAGACGCTCC AATCTCTGTC CCAGGTGTCT  
5851 AACGAATAAA TAGGTAAAAG AAAAA

**FIG. 2A**

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Predicted amino acid sequence of rat NaN (1765 a.a).

1 MEERYYPVIF PDERNFRPFT SDSLAAIEKR IAIQKERKKS KDKAAAEPQP  
 51 RPQLDLKASR KLPKLYGDIP PELVAKPLED LDPFYKDHKT FMVLNKKRTI  
 DI-S1  
 101 YRFSAKRALF ILGPFNPLRS LMIRISVHSV FSMFIICTVI INCMFMANS  
 DI-S2 DI-S3  
 151 ERSFDNDIPE YVFIGIYILE AVIKILARGE IVDEFSFLRD PWNWLDIFI  
 DI-S4  
 201 GTAIATCFPG SQVNLSALRT FRVFRALKAI SVISGLKVIV GALLRSVKKL  
 DI-S5  
 251 VDVMVLTLEC LSIFALVGOO LFMGILNQKC IKHNCGPNPA SNKDCFEKEK  
 DI-SS1  
 301 DSEDFIMCGT WLGSRPCPNG STCDKTTLNP DNNYTKFDNE GWSFLAMFRV  
 DI-SS2 DI-S6  
 351 MTODSWERLY RQILRTSGIY FVFFFVVVIF LGSFYLLNLT LAVVTMAYEE  
 401 QNRNVAAETE AKEKMFOEAQ QLLREEKEAL VAMGIDRSSL NSLQASSFSP  
 451 KKRKFEGSKT RKSFFMRGSK TAQASASDSE DDASKNPQLL EQTKRLSQNL  
 501 PVDLFDEHVD PLHRQRALSA VSILTITMQE QEKFOEPCFP CGKNLASKYL  
 DII-S1  
 551 VWDCSPQWLC IKKVLRTIMT DPTELAITI CIIINTVFLA VEHNMDDNL  
 DII-S2 DII-S3  
 601 KTILKIGNWV FTGIFIAEMC LKIIALDPYH YFRHGWNVFD SIVALLSLAD  
 DII-S4  
 651 VLYNTLSDDN RSFLASLRVL RVFKLAKSWP TLNTLIKIIG HSVGALGNLT  
 DII-S5 DII-SS1  
 701 VVLTIVVFIF SVVGMRLFGT KFNKTAYATQ ERPRRRWHMD NEYHSFLVVF  
 DII-SS2 DII-S6  
 751 RILCGEWIEN MWGCMQDMDG SPLCIIVFVL IMVIGKLVVL NLFIALLLNS

**FIG. 2B**

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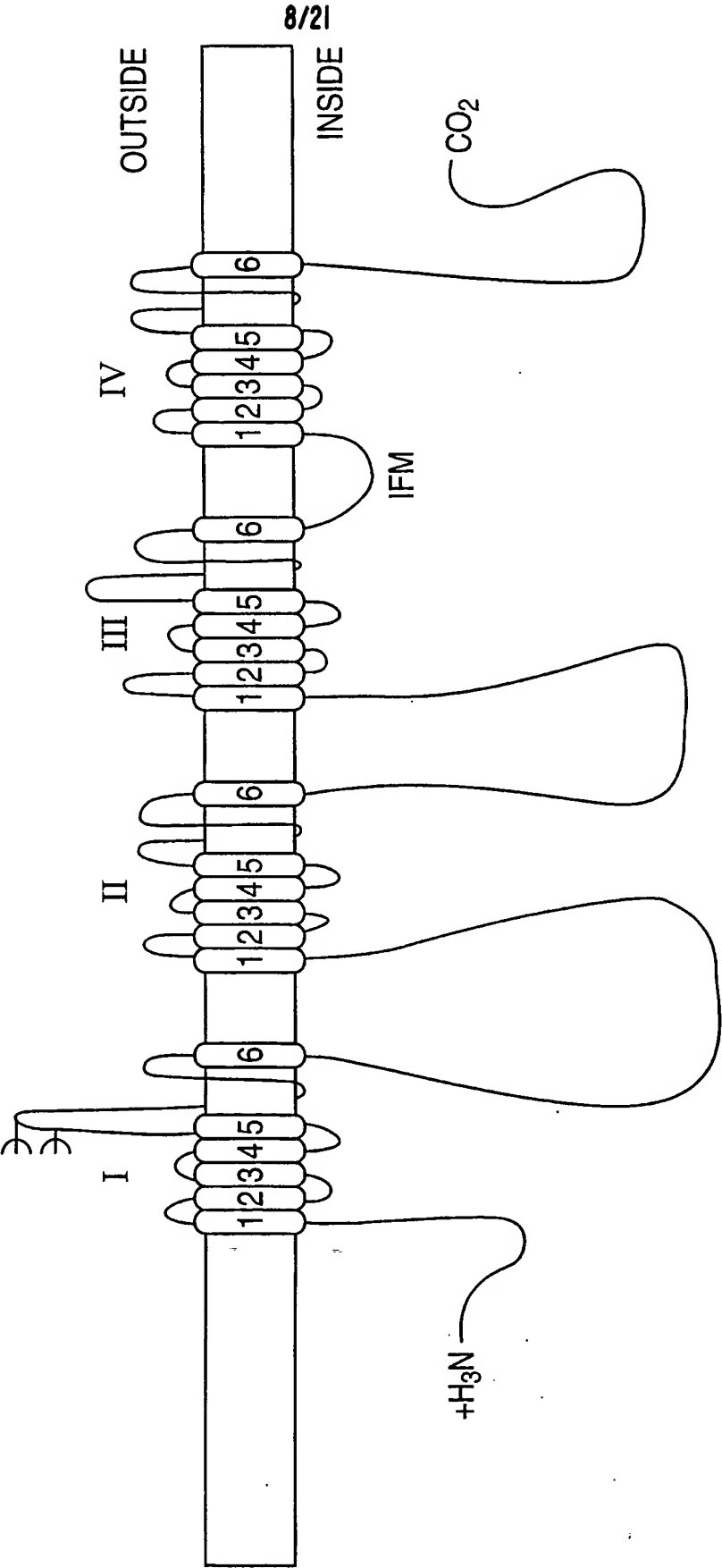
801 FSNEEKDGSL EGETRKTQVQ LALDRFRRAF SFMLHALQSF CCKKCRRKNS  
 851 PKPKETTESF AGENKDSILP DARPWKEYDT DMALYTGQAG APLAPLAEVE  
 901 DDVEYCGEGG ALPTSQHSAG VQAGDLPPET KQLTSPDDQG VEMEVFSEED  
 951 LHLSIQSPRK KSDAVSMLSE CSTIDLNDIF RNLQKTVSPK KQPDRCFPKG  
 DIII-S1  
 1001 LSCHFLCHKT DKRKSPWWLW WNIRKTCYQI VKHSWFESFI IFVILLSSGA  
 DIII-S2  
 1051 LIFEDVNLPS RPQVEKLLRC TDNIFTFIFL LEMILKWVAF GFERRYFTSAW  
 DIII-S3 DIII-S4  
 1101 CWLDFLIVVV SVLSLMNLPS LKSFRTLRL RPLRALSOFE GMKVVVYALI  
 DIII-S5  
 1151 SAIPAILNVL LVCLIFWLVF CILGVNLFSG KFGRCINGTD INMYLDFTEV  
 DIII-SS1 DIII-SS2  
 1201 PNRSQCNISN YSWKVPQVNF DNVGNAYLAL LOVATYKGWL EIMNAAVDSR  
 DIII-S6  
 1251 EKDEQPDFEA NLYAYLYFVV FIIFGSFFTL NLFIGVIIDN FNQQQKKLGG  
 DIV-S1  
 1301 QDIFMTEEQK KYYNAMKKLG TKKPQKPIPR PLNRCQAFVF DLVTSHVEDV  
 1351 IILGLIVLNM IIMMAESADQ PKDVKKTFDI LNIAFVVIFT IECLIKVFAL  
 DIV-S4  
 1401 RQHYFTNGWN LFDCVVVVLS IISTLVSRL ESDISFPPTL FRVVRLARIG  
 DIV-S5  
 1451 RILRLVRAAR GIRTLFLFALM MSLPSLFNIG LLLFLVMFIY AIFGMSWFSK  
 DIV-SS1 DIV-SS2  
 1501 VKKGSGIDDI FNFETFTGSM LCLFOITTS AWDTLLNPML EAKEHCNSSS  
 DIV-S6  
 1551 QDSCQQPOIA VVYFVSYIII SFLIVVNMYI AVILENFNTA TEESEDPLGE  
 1601 DDFFEIFYEVW EKFDPEASQF IQYSALSDFA DALPEPLRVA KPNKQOFLVM

***FIG. 2C***

1701 YEPIVTTTKR KEEEQGAIVI QRAYRKHMEK MVKLRLKDRS SSSHQVFCNG

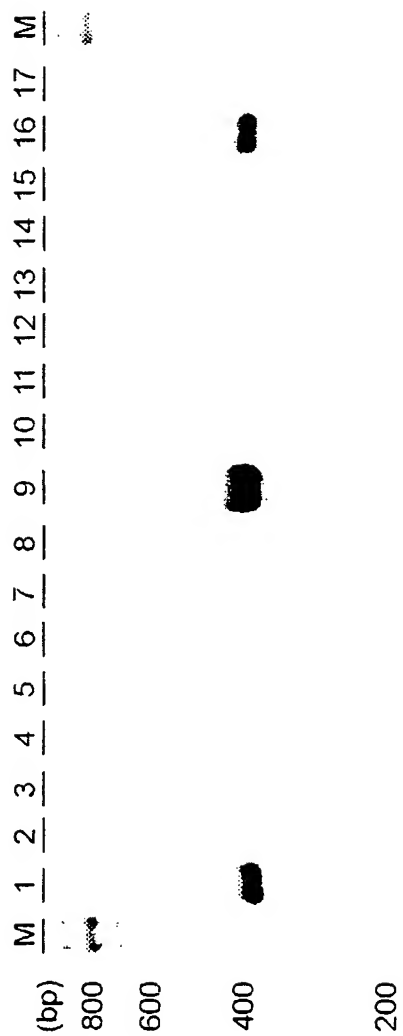
1751 DLSSLDVAKV KVNND\*

**FIG. 3**  
VOLTAGE-GATED SODIUM CHANNEL  $\alpha$  SUBUNIT



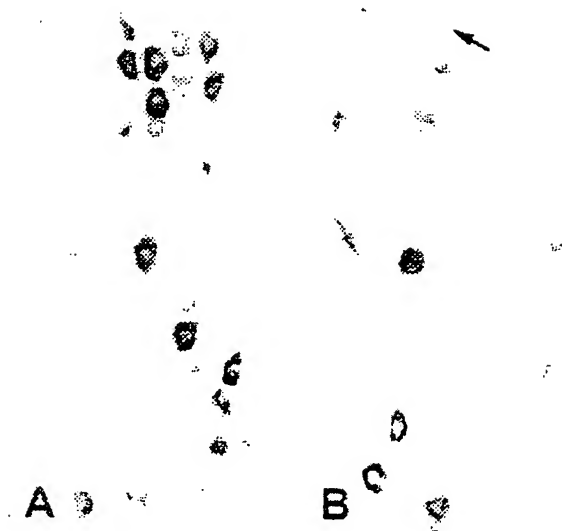
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**FIG. 4**



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**FIG. 5**



C — D

E F —

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**FIG. 6**

RESTRICTION ENZYME ANALYSIS OF  $\alpha$ -SUBUNIT PCR PRODUCTS FROM DOMAIN I USING THE FOLLOWING PRIMERS: NACHD1A.1-4 (FORWARD PRIMERS) AND NAAAGEN.REV1-3 (REVERSE PRIMERS).

Generic Primer pair	F1 R.1	F2 R1	F1 R1	F1/F3 R1	F1 R1	F1/F3 R1	F1 R1	F1/F3 R1	F1 R1	F2 R2	F4 R3	F2 R3
	$\alpha$ I 558 bp	$\alpha$ II 561 bp	$\alpha$ III 561 bp	$\alpha$ VI 507 bp	$\alpha$ rPN1 501 bp	$\alpha$ rH1 518 bp	$\alpha\mu$ 1 602 bp	$\alpha$ SNS 479 bp	$\alpha$ NaG 501 bp	$\alpha$ NaN 468 bp		
EcoRV	+	-	-	-	-	-	-	-	-	-	-	-
EcoNI	-	+	-	-	-	-	-	-	-	-	-	-
AvaI	-	-	+	-	-	-	-	-	-	-	-	-
SphI	-	-	-	+	-	-	-	-	-	-	-	-
BamHI	-	-	-	-	+	-	-	+	-	-	-	-
AccI	-	-	-	-	-	+	-	-	-	-	+	185, 283
NgoMI	-	-	-	-	-	-	+	-	+	-	95, 406	-
AflII	-	-	-	-	-	-	-	+	-	224, 255	-	-
XbaI	-	-	-	-	-	-	-	-	+	-	165, 336	-
EcoRI	-	-	-	-	-	-	-	-	-	-	-	+
												214, 254



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**FIG. 7A-1**

Sequence of the mouse NaN cDNA.

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1      TCTGAGCCAA GGGTGAAGAT GGAGGAGAGG TACTATCCAG TGATCTTCCC AGACGAGAGG
61     AATTTCGGCC CCTTCACTTT CGACTCTTTG GCTGCAATAG AGAAGCGGAT CACCATCCAA
121    AAGGAGAAGA AGAAATCCAA AGACAAGGCA GCAACTGAGC CCCAGCCTCG GCCTCAGCTC
181    GACCTAAAGG CCTCCAGGAA GTTACCTAAG CTCTATGGCG ACGTTCCCCC TGACCTTATA
241    GCGAAGCCCC TGGAAGATCT GGACCCATTT TACAAAGACC ATAAGACATT CATGGTATTG
301    AACAAGAAGA GAACAATCTA TCGCTTCAGC GCCAAGAGGG CCTTGTTTCAT TCTGGGGCCT
361    TTTAATCCCA TCAGAAGCTT CATGATTGCG ATCTCTGTCC ATTCAGTCTT CAGCATGTTC
421    ATTATCTGCA CAGTGATCAT CAACTGTATG TTCATGGCTA ATAATTCTTC TGTGGACAGT
481    CGTCCTAGCA GTAACATTCC CGAATACGTC TTCATTGGGA TTTATGTTTT AGAAGCTGTG
541    ATTTAAATAT TGGCAAGAGG CTTCAATTGT GATGAGTTTT CCTACCTCCG AGATCCTTGG
601    AACTGGCTGG ACTTCATTGT CATCGGAACA GCGATAGCGC CTTGTTTTCT CGGTAACAAA
661    GTCAATAATC TTTCCACTCT ACGTACCTTC CGAGTGTGGA GAGCTCTGAA AGCCATTTCT
721    GTAATCTCAG GTCTGAAGGT CATCGTGGGT GCCCTGCTGC GCTCCGTGAA GAAGCTAGTG
781    GACGTGATGG TCCTCACTCT CTTTTCCTC AGCATCTTTG CCCTGGTTGG TCAGCAGCTC
841    TTCATGGGAA TTCTGAGCCA GAAATGTATT AAGGACGACT GTGGCCCTAA CGCTTTTTTC
901    AACAAGGATT GCTTTGTAAA AGAAAATGAT AGCGAGGACT TCATAATGTG TGGCAACTGG
961    CTCGGCAGAA GATCCTGCCC CGATGGTTCC ACGTGCAATA AAACCACATT TAACCCAGAT
1021   TATAATTATA CAACTTTGA CAGCTTTGGC TGGTCTTTTC TCGCCATGTT CCGGGTTATG
1081   ACTCAAGACT CCTGGGAGAA GCTTTATCGA CAGATCCTTC GCACCTCCGG GATCTACTTT
1141   GTCTTCTTCT TCGTGGTCGT CATCTTCCTG GGCTCTTTCT ACCTGCTTAA CTTAACCCCTG
1201   GCTGTCTGTA CCATGGCTTA CGAGGAACAG AACAGAAATG TCGCTGCCGA GACAGAGGCC
1261   AAGGAGAAGA TGTTTCAGGA AGCCGAGCAG CTGTTGAGGG AGGAAAAGGA GGCTCTGGTT
1321   GCCATGGGAA TTGACAGAAC TTCCCTTAAT TCCCTCCAAG CTTCTGCTTT TTCCCCAAG
1381   AAGAGGAAGT TTTTGGCAG TAAGACAAGA AAGTCCTTCT TTATGAGAGG GTCCAAGACA
1441   GCCCCGAGCCT CAGCGTCCGA TTCAGAGGAC GATGCCTCTA AAAACCCACA ACTCCTTGAG
1501   CAAACAAAAC GACTATCCCA GAACTTGCCC GTAGAACTCT TTGATGAGCA CGTGGACCCC
1561   CTCCATAGGC AGAGAGCGCT GAGTGCCGTC AGTATCTTAA CCATCACCAT GCAGGAACAA
1621   GAAAAATCCC AGGAGCCTTG TTTCCCGTGT GGGAAAAACT TGGCATCCAA GTACCTGGTG
1681   TGGGAATGTA GCCCTCCGTG GCTGTGCATA AAGAAGGTCC TGCAGACTAT CATGACAGAC
1741   CCTTCACTG AGCTGGCCAT CACCATCTGC ATCATCGTCA ATACTGTCTT CTTGGCCATG
1801   GAACACCACA ATATGGATAA CTCTTTAAAA GACATACTGA AAATAGGAAA CTGGGTTTTT
1861   ACTGGAATTT TCATAGCGGA AATGTGTCTC AAGATCATTG CGCTAGACCC TTACCACTAC
1921   TTCCGGCACG GCTGGAACAT CTTTGACAGC ATTGTGGCCC TTGTGAGTCT CGCTGACGTG

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**FIG. 7A-2**

1981 CTCTTCCACA AACTGTCTAA AAACCTCTCC TTCTTGGCTT CCCTCAGAGT GCTGAGGGTC  
2041 TTCAAGTTAG CCAAATCCTG GCCCACATTA AACACTCTCA TTAAGATCAT CGGCCACTCC  
2101 GTGGGTGCGC TCGGAAACCT GACTGTGGTC CTAACGATCG TGGTCTTCAT CTTTTCCGTG  
2161 GTTGGCATGC GGCTCTTTGG TGCCAAGTTT AACAAGACTT GCTCCACCTC TCCGGAGTCC  
2221 CTCCGGCGCT GGCACATGGG TGATTTCTAC CATTCTTCC TGGTGGTGTT CCGCATCCTC  
2281 TGTGGGGAGT GGATCGAGAA CATGTGGGAA TGCATGCAGG AGATGGAAGG CTCCCCGCTG  
2341 TGTGTCATCG TCTTTGTGCT GATCATGGTG GTCGGGAAGC TCGTGGTGCT TAACCTCTTC  
2401 ATTGCCTTGC TGCTCAATTC CTTCAGCAAT GAGGAAAAGG ATGGGAACCC AGAAGGAGAG  
2461 ACCAGGAAAA CCAAAGTGCA GCTAGCCCTG GATCGGTTCA GCCGAGCGTT CTACTTCATG  
2521 GCGCGCGCTC TTCAGAATTT CTGTTGCAAG AGATGCAGGA GGCAAACTC GCCAAAGCCA  
2581 AATGAGGCAA CAGAAAGCTT TGCTGGTGAG AGTAGAGACA CAGCCACCCT GGATACAAGG  
2641 TCCTGGAAGG AGTATGATTC AGAAATGACT CTGTACACTG GGCAGGCCCG GGCTCCACTG  
2701 GCCCCACTGG CAAAAGAAGA GGACGATATG GAATGTTGTG GTGAATGTGA TGCCTCACCT  
2761 ACCTCACAGC CTAGTGAGGA AGCTCAGGCC TGTGACCTCC CTCTGAAGAC CAAGCGGCTC  
2821 CCCAGCCCAG ATGACCACGG GGTTGAAATG GAAGTGTTTT CCGAAGAAGA TCCGAATTTA  
2881 ACCATACAGA GTGCTCGAAA GAAGTCTGAT GCGGCAAGCA TGCTCTCAGA ATGCAGCACA  
2941 ATAGACCTGA ATGATATCTT TAGAAATTTA CAGAAAACAG TTTCCCCCA AAAGCAACCA  
3001 GATCGATGCT TTCCCAAGGG CCTCAGTTGT ATCTTTCTAT GTTGCAAAAC AATCAAAAAA  
3061 AAGTCCCCCT GGGTCTGTG GTGGAATCTT CGGAAAACCT GCTACCAAT CGTGAAGCAT  
3121 AGCTGGTTTG AGAGCTTCAT AATTTTTGTC ATCCTGCTGA GCAGCGGAGC ACTGATATTC  
3181 GAAGATGTCA ATCTTCCCAG CCGGCCCAA GTTGAAAAAT TACTGAAGTG TACCGATAAT  
3241 ATTTTCACAT TTATTTTCTT CCTGGAATG ATTTTGAAGT GGGTGGCCTT TGGATTCCGG  
3301 AAGTATTTCA CCAGTGCTG GTGCTGGCTC GATTTCTCA TTGTGGTGGT GTCTGTGCTC  
3361 AGCCTCACGA ACTTACCAA CTTGAAGTCC TTCCGGAATC TGCGAGCGCT GAGACCTCTG  
3421 CGGGCACTGT CTCAGTTTGA AGGAATGAAG GTTGTGTCA ATGCCCTCAT GAGTGCCATA  
3481 CCTGCCATCC TCAATGTCTT GCTGGTCTGC CTCATTTTCT GGCTCATATT TTGTATCCTG  
3541 GGAGTAAATT TTTTCTGGA GAAGTTTGGA AGATGCATTA ATGGAACAGA CATAAATAAA  
3601 TATTTCAACG CTTCCAATGT TCCAAACCA AGCCAATGTT TAGTTAGTAA TTACACGTGG  
3661 AAAGTCCCGA ATGTCAACTT TGACAACGTG GGGAAATGCCT ACCTTGCCCT GCTGCAAGTG  
3721 GCGACCTATA AGGGCTGGCT GGACATTATG AATGCAGCTG TTGATTCCAG AGGGAAAGAT  
3781 GAGCAGCCGG CCTTTGAGGC GAATCTATAC GCATACCTTT ACTTCGTGGT TTTTATCATC  
3841 TTCGGCTCAT TCTTTACCCT GAACCTCTTT ATCGGTGTTA TTATTGACAA CTTCAATCAG  
3901 CAGCAGAAAA AGTTAGGTGG CCAAGACATT TTTATGACAG AAGAACAGAA GAAATATTAC  
3961 AATGCAATGA AAAAGTTAGG AACCAAGAAG CCTCAAAAGC CCATCCCAAG GCCCCTGAAC  
4021 AAATGTCAAG CCTTCGTGTT CGATTGGTC ACAAGCCAGG TCTTTGACGT CATCATCTG  
4081 GGTCTTATTG TCACAAACAT GATTATCATG ATGGCTGAAT CTGAAGGCCA GCCCAACGAA  
4141 GTGAAGAAAA TCTTTGATAT TCTCAACATA GTCTTCGTGG TCATCTTTAC CGTAGAGTGT

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**FIG. 7A-3**

4261 TGTGTGGTCG TGGTCTTTC CATCATTAGT ACCTTGGTTT CTGGCTTGGA GAACAGCAAC  
4321 GTCTTCCCGC CCACACTCTT CAGGATTGTC CGCTTGGCTC GGATCGGTCG AATCCTCAGA  
4381 CTGGTCCGGG CGGCTCGAGG AATCAGGACA CTCCTTTTCG CGTTGATGAT GTCTCTCCCC  
4441 TCTCTCTTCA ACATTGGTCT GCTTCTCTTT CTGGTGATGT TCATTTATGC CATCTTTGGG  
4501 ATGAACTGGT TTTCCAAAGT GAAGAGAGGC TCTGGGATTG ATGACATCTT CAACTTTGAC  
4561 ACTTTCTCGG GCAGCATGCT CTGCCTCTTC CAGATAACCA CTTCAGCCGG CTGGGATGCT  
4621 CTCCTCAACC CCATGCTGGA ATCAAAAGCC TCTTGCAATT CCTCCTCCCA AGAGAGCTGT  
4681 CAGCAGCCGC AGATAGCCAT AGTCTACTTC GTCAGCTACA TCATCATCTC CTTTCTCATT  
4741 GTGGTTAACA TGTACATAGC TGTGATTCTA GAGAACTTCA ACACAGCCAC AGAGGAGAGC  
4801 GAGGACCCCC TGGGCGAAGA CGACTTTGAG ATCTTCTATG AGATCTGGGA GAAGTTTGAC  
4861 CCCGAAGCAA CACAGTTCAT CCACTACTCA TCCCTCTCTG ACTTCGCCGA CGCCCTGCCC  
4921 GAGCCGTTGC GTGTGGCCAA GCCCAACAGG TTTCAGTTTC TCATGATGGA CTTGCCCATG  
4981 GTGATGGGTG ATCGCCTCCA TTGCATGGAT GTTCTCTTTG CTTTCACCAC CAGGGTCCTC  
5041 GGGAACTCCA GCGGCTTGGA TACCATGAAA GCCATGATGG AGGAGAAGTT CATGGAGGCC  
5101 AATCCTTTCA AGAAGTTGTA CGAGCCCATT GTCACCACCA CAAAGAGGAA GGAGGAGGAG  
5161 GAATGTGCCG CTGTCATCCA GAGGCGCTAC CGGAGACACA TGGAGAAGAT GATCAAGCTG  
5221 AAGCTGAAAAG GCAGGTCAAG TTCATCGCTC CAGGTGTTTT GCAATGGAGA CTTGTCTAGC  
5281 TTGGATGTGC CCAAGATCAA GGTTCATTGT GACTGAAACC CCCACCTGCA CGCCTACCTC  
5341 ACAGCCTCAC AGCTCAGCCC CCAGCCTCTG GCGAACAAGC GGCGGACTCA CCGAACAGGC  
5401 CGTTCAACTT GTTTTTTTGG GTGAAAGAGG TGATAGGTTG GTGTCCATTT TTAAATGATT  
5461 CTTGGAAAGA TTGAACGTCG GAACATGTTA GAAAGGACTG CCAAGGACAT CCACAGTAAC  
5521 GGAAGGCCTG AAGGACAGTT CAAATTATGT AAAGAAACGA GAAGGAAAGG TCACATGTCT  
5581 GTTCAGTTTT AAGTATGTGA CCTGCCACAT GTAGCTCCTT TGCATGTTAA GTGAGAAGTC  
5641 AAAACCCTGC CATAAGTAAA TAGCTTTGTT GCAGGTGTTT CTACCACTGC TCGGATTTG  
5701 GGTGTATGGC TCAAACCTGA AAGCATGACT CTGACTTGTC AGCACCCEAA CTTTCAGAAG  
5761 CTCTGATCTC TGTCTAGGT GTTTGACAAA TAAATACATA AAANAAAAAA AAAAAAAAAA  
5821 AA

**FIG. 7B-1**

**22.540 Charge at PH 7.0**

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**FIG. 7B-2**

781 LIMVVGKLVV LNLFIALLLN SFSNEEKDGN PEGETRKTIV QLALDRFSRA FYFMARALQN  
 DII-S6  
 841 FCCKRCRRQN SPKPNEATES FAGESRDTAT LDTRSWKEYD SEMTLYTGQA GAPLAPLAKE  
 901 EDDMECCGEC DASPTSQPSE EAQACDLPLK TKRLPSPDDH GVEMEVFSEE DPNLTIQSAR  
 961 KKSDAASMLS ECSTIDLNDI FRNLQKTVSP QKQPDRCFFK GLSCIFLCKK TIKKKSPPWL  
 1021 WWNLKRTCQY IVKHSWFESF IIFVILLSSG ALIFEDVNLP SRPQVEKLLK CTDNIETEIF  
 DIII-S1 DIII-S2  
 1081 LLEMILKHVA EGFERKYFTSA WCWLDELIVV VSVLSLTNLP NLKSFRLRA LRPLRALSOE  
 DIII-S2 DIII-S3 DIII-S4  
 1141 EGMKVVVNAL MSAIPAILNV LLVCLIFWLI FCILGVNFES GKFGRCINGT DINKYFNASH  
 DIII-S5  
 1201 VPNQSQCLVS NYTWKVPNVN FDNVGNAYLA LLQVATYKGV LDIMNAAVDS RKGDEQPAFE  
 DIII-S51 DIII-S52  
 1261 ANLYAXLYFV VPIIEGSEFT LNLFIGVID NFNQQQKGLG GODIEMTEEQ KKYNNAMIKL  
 DIII-S6  
 1321 GTGQPQKPIP RPLNKCQAFV FDLVTSQVED VILGLIVTM MIIMMAESEG QPNEVKKIPD  
 DIV-S1  
 1381 ILNIVEVWIF TVECLIKVEA LRQHYFTNGH NLFDCVWVL SIISTLVSGL ENSNVFPPTL  
 DIV-S2 DIV-S3  
 1441 FRIVRLARIG RILRLVRAAR GIRTLLPALM MSLPSLFNIG LLLFLVMFIY AIEGMWESK  
 DIV-S4 DIV-S5  
 1501 VKRGSGIDDI FNEFTESGSM LCLFOITTS GWDALLNPML ESKASCNSSS QESCQPOIA  
 DIV-S51 DIV-S52  
 1561 IVYFVSXIII SELIVVNMVI AVILENFNTA TEESDPLGE DDFEIFYEIV EKFDPEATQF  
 DIV-S6  
 1621 IQYSSLSDFD DALPEPLRVA KPNRFQFLMM DLPVMVGDRL HCMOVLFAPT TRVLGNSSGL  
 1681 DTMKAMMEEK FMEANPFKCL YEPVITTTKR KEEEBCAAVI QRAYRRHMEK MIKCLKGERS  
 1741 SSSLOVFCNG DLSSLDVPKI KVHCD.

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**FIG. 8A-1** Partial Human NaN Nucleotide Sequence

TCCATTGTCATTGGAATAGCGATTGTGTCATATATTCCAGGAATCACCATCAAACCTATTGCCCC  
TGCGTACCTTCCGTGTGTTTCAGAGCTTTGAAAGCAATTCAGTAGTTTCACGTCTGAAGGTCAT  
CGTGGGGGCCTTGCTACGCTCTGTGAAGAAGCTGGTCAACGTGATTATCCTCACCTTCTTTTGC  
CTCAGCATCTTTGCCCTGGTAGGTCAGCAGCTCTTCATGGGAAGTCTGAACCTGAAATGCATCT  
CGAGGGACTGTAAAAATATCAGTAACCCGGAAGCTTATGACCATTGCTTTGAAAAGAAAAGAAA  
ATTACCTGAATTCAAAATGTGTGGCATCTGGATGGGTAAACAGTGCCTGTTCCATACAATATGA  
ATGTAAGCACACCAAAATTAATCCTGACTATAATTATACGAATTTTGACAACCTTTGGCTGGTCT  
TTTCTTGCCATGTTCCGGCTGATGACCAAGATTCTGGGAGAAGCTTTATCAACAGACCCTGC  
GTACTACTGGGCTCTACTCAGTCTTCTTCTTCATTGTGGTCATTTTCTGGGCTCCTTCTACCTGA  
TTAACTTAACCTGGCTGTTGTTACCATGGCATATGAGGAGCAGAACAAGAATGTAGCTGCAG  
AGATAGAGGCCAAGGAAAAGATGTTTCAGGAAGCCCAGCAGCTGTTAAAGGAGGAAAAGGAG  
GCTCTGGTTGCCATGGGAATTGACAGAAGTTCACCTTACTTCCCTTGAAACATCATATTTTACCC  
CAAAAAAGAGAAAAGCTCTTTGGTAATAAGAAAAGGAAGTCCTTCTTTTGAGAGAGTCTGGGA  
AAGACCAGCCTCCTGGGTGAGATTCTGATGAAGATTGCCAAAAAAGCCACAGCTCCTAGAGC  
AAACCAAAACGACTGTCCAGAATCTATCAYTGGACCACTTTGATGAGCATGGAGATCCTCTCCA  
AAGGCAGAGAGCACTGAGTGCTGTCAGCATCCTCAACCATCAACATGAAGGAACAAGAAAAATC  
ACAAGAGCCTTGTCTCCCTTGTGGAGAAAACCTGGCATCCAAGTACCTCGTGTGGAACCTGTTGC  
CCCCAGTGGCTGTGCGTTAAGAAGGTCCTGAGAACTGTGATGACTGACCCGTTTACTGAGCTGG  
CCATCAACCATCTGCATCATCATCAACACTGTCTTCTTGGCCATGGAGCATCAAGATGGAGGC  
CAGTTTTGAGAAGATGTTGAATATAGGGAATTTGGTTTTCACTAGCATTTTTATAGCAGAAAATG  
TGCCTA AAAATCATTGCGCTCGATCCCTACCACTACTTTCGCCGAGGCTGGAACATTTTTGACA  
GCATTGTTGCTCTTCTGAGTTTTGCAGATGTAATGAACTGTGTACTTCAAAAAGAGAAGCTGGCC  
ATTCTTGCGTTCCCTCAGAGTGCTCAGGGTCTTCAAGTTAGCCAAATCCTGGCCAACTTTGAAC  
ACACTAATTAAGATAATCGGCAACTCTGTGCGAGCCCTTGGAAGCCTGACTGTGGTCTGGTCA  
TTGTGATCTTTATTTTCTCAGTAGTTGGCATGCAGCTTTTTGGCCGTAGCTTCAATTCCCAAAAG  
AGTCCAAAACCTCTGTAACCCGACAGGCCGACAGTCTCATGTTTACGGCACTGGCACATGGGG  
GATTTCTGGCACTCCTTCTAGTGGTATTCCGCATCCTCTGCGGGGAATGGATCGAAAATATGT  
GGGAATGTATGCAAGAAGCGAATGCATCATCATCATTGTGTGTTATTGTCTTCATATTGATCAC  
GGTGATAGGAAAACCTGTGGTGCTCAACCTCTTCATTGCCTTACTGCTCAATTCCTTTAGCAAT  
GAGGAAAGAAAATGGAAACTTAGAAGGAGAGGCCAGGAAAACTAAAGTCCAGTTAGCACTGGA  
TCGATTCCGCCGGGCTTTTTGTTTTGTGAGACACACTCTTGAGCATTTCTGTCACAAGTGGTGCA  
GGAAGCAAAACTTACCACAGCAAAAAGAGGTGGCAGGAGGCTGTGCTGCACAAAGCAAAAGAC  
ATCATTCCCTGGTCATGGAGATGAAAAGGGGCTCAGAGACCCAGGAGGCTTGGTATACTA  
ACCTCTGTACCAAGACCCTGGGCGTCAGGCATGATTGGACTTGGTTGGCACCCTTGCGGAG  
GAGGAAGATGACGTTGAATTTCTGGTGAAGATAATGCACAGCGCATCACACAACCTGAGCCT  
GAACAACAGGCCTATGAGCTCCATCAGGAGAACAAGAACCCACGAGCCAGAGAGTTCAAAG  
TGTGGAATTTGACATGTTCTCTGAAGATGAGCCTCATCTGACCATAACAGGATCCCCGAAAGAA  
GTCTGATGTTACCAGTATACTATCAGAATGTAGCACCATTGATCTTCAGGATGGCTTTGGATGG  
TTACCTGAGATGTTTCCCAAAAAGCAACCAGAGAGATGTTTGGCCAAAGGCTTTGGTTGCTGCT

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**FIG. 8A-2**

CCTGCTACCAAATAGTGAAACACAGCTGGTTTGAGAGCTTTATTATCTTTGTGATTCTGCTGAG  
CAGTGGGGCACTGATATTTGAAGATGTTACCTTGAGAACCAACCCAAAATCCAAGAATTACT  
AAATTGTA CTGACATTATTTTACACATATTTTATCCTGGAGATGGTACTAAAATGGGTAGCC  
TTCGATTTGGAAAAGTATTTACCAGTGCCTGGTGCTGCCTTGATTTTCATCATTGTGATTGTCTC  
TGTGACCACCTCATTAACCTTAATGGAATTGAAGTCCTCCGGACTCTACGAGCACTGAGGCCT  
CTTCGTGCGCTGTCCAGTTTGAAGGAATGAAGGTGGTGGTCAATGCTCTCATAGGTGCCATAC  
CTGCCATTCTGAATGTTTTGCTTGTCTGCCTCATTTTCTGGCTCGTATTTTGTATTCTGGGAGTAT  
ACTTCTTTTCTGGAAAATTTGGGAAATGCATTAATGGAACAGACTCAGTTATAAATTATACCAT  
CATTACAAATAAAAGTCAATGTGAAAGTGGCAATTTCTCTTGGATCAACCAGAAAAGTCAACTTT  
GACAATGTGGGAAATGCTTACCTCGCTCTGCTGCAAGTGGCAACATTTAAGGGCTGGATGGAT  
ATTATATATGCAGCTGTTGATTCCACAGAGAAAGAACAACAGCCAGAGTTTGAGAGCAATTCA  
CTCGGTTACATTTACTTCGTAGTCTTTATCATCTTTGGCTCATTCTTCACTCTGAATCTCTTCATT  
GGCGTTATCATTGACAACTTCAACCAACAGCAGAAAAAGTTAGGTGGCCAAGACATTTTTATG  
ACAGAAGAACAGAAGAAATACTATAATGCAATGAAAAAATTAGGATCCAAAAAACCTCAAAA  
ACCCATTCCACGGCCTCTGAACAAATGTCAAGGTCTCGTGTTCGACATAGTCACAAGCCAGATC  
TTTGACATCATCATATAAGTCTCATTATCCTAAACATGATTAGCATGATGGCTGAATCATACA  
ACCAACCCAAAGCCATGAAATCCATCCTTGACCATCTCAACTGGGTCTTTGTGGTCATCTTTAC  
GTTAGAATGTCTCATCAAAATCTTTGCTTTGAGGCAATACTACTTCACCAATGGCTGGAATTTA  
TTTGA

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**FIG. 8B****Partial Human NaNAmino Acid Sequence**

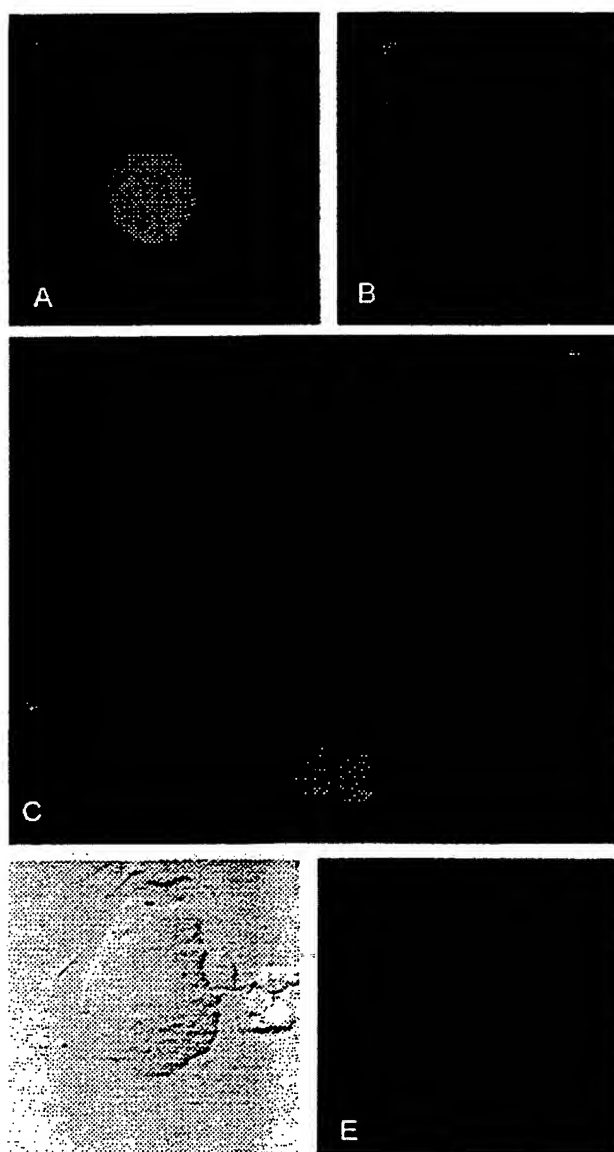
SI VIGIAIVSYI PGITIKLLPL RTRVFRALK AISVVSRLKV IVGALLRSVK KLVNVIIITF  
FCLSIFALVG QQLFMGSLNL KCISRDCKNI SNPEAYDHCF EKKENSPEFK MCGIWMGNSA  
CSIQYECKHT KINPDYNYTN FDNFGWSFLA MFRLMTQDSW EKLYQQTLRT TGLYSVFFFI  
VVIFLGSFYI INLTAVVTM AYEEQNKVA AEIEAKEKMF QEAQQLLEE KEALVAMGID  
RSSLTSELS YFTPCKRKL FGNKKRSFFL RESGKDQPPG SDSDEDCQKK PQLLEQTKRL  
SQNLSLDHFD EHGDPQRQR ALSAVSILTI TMKEQEKSE PCLPCGENLA SKYLVWNCCP  
QWLCVKKVLR TVMTDPFTEL AITICIIINT VFLAMEHHKM EASF EKMLNI GNLVFTSIFI  
AEMCLKIAL DPYHYFRGW NIFDSIVALL SFADVMNCVL QKRSWPFLRS  
FRVLRVFKLAKSWPTLNTLI KIIGNSVGAL GSLTVVLVIV IFISVVGMMQ LFGRSFNSQK  
SPKLCNPTGP TVSCLRHWHM GDFWHSFLVV FRILCGEWIE NMWECMQEAN ASSSLCVTVF  
ILITVIGKLV VLNLFIALLL NSFSNEERNNG NLEGEARKTK VQLALDRFR AFCEVRHTLE  
HFCHKWCRKQ NLPQKQEVAG GCAAQSKDII PLVMEMKRGSE ETQEELGILT SVPKTLGVRH  
DWTWLAPLAE EEDDVEFSGE DNAQRITQPE PEQQAYELHQ ENKKPTSQRVQSVEIDMFSE  
DEPHLTIQDP RKKSDVTSIL SECSTIDLQD GFGWLPEMVP KKQPERCLPK GFGCCFPCCS  
VDKRPKPPWVI WWNLRKTCYQ IVKHSWFESF IIFVILLSSG ALIFEDVHLE NQPKIQELLN  
CTDIIFTHIF ILEMVLKWVA FGFGKYFTSA WCCLDFIIVI VSVTTILNLM ELKSFRTLRA  
LRPLRALSQF EGMKVVVNAL IGAIPAILNV LLVCLIFWLV FCILGVYFFS GKFGKCKINGT  
DSVINYTIIT NKSQCESGNF SWINQKVNFD NVGNAYLALL QVATFKGWMD IYAAVDSTE  
KEQQPEFESN SLGYTYFVVF IIFGSFFTLN LFIGVIIDNF NQQQKKLGGQ DIFMTEEQKK  
YYNAMKKLGS KKPQKPIPRP LNKCQGLVFD IVTSQIFDII IISLIILNMI SMMAESYNQP  
KAMKSILDHL NWVFVVIFTL ECLIKIFALR QYYFTNGWNL FDCVVVLLSIV



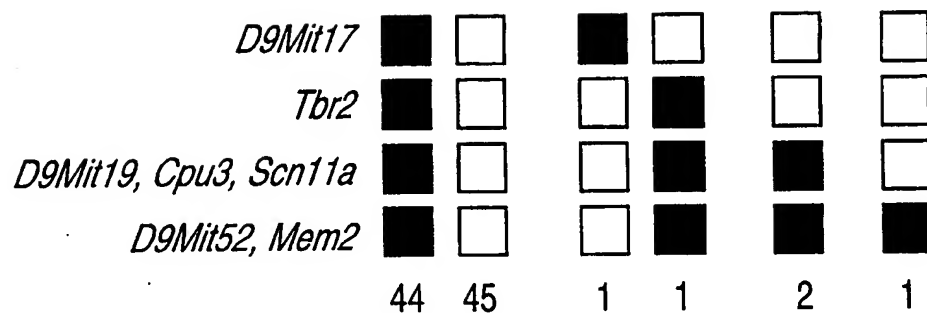
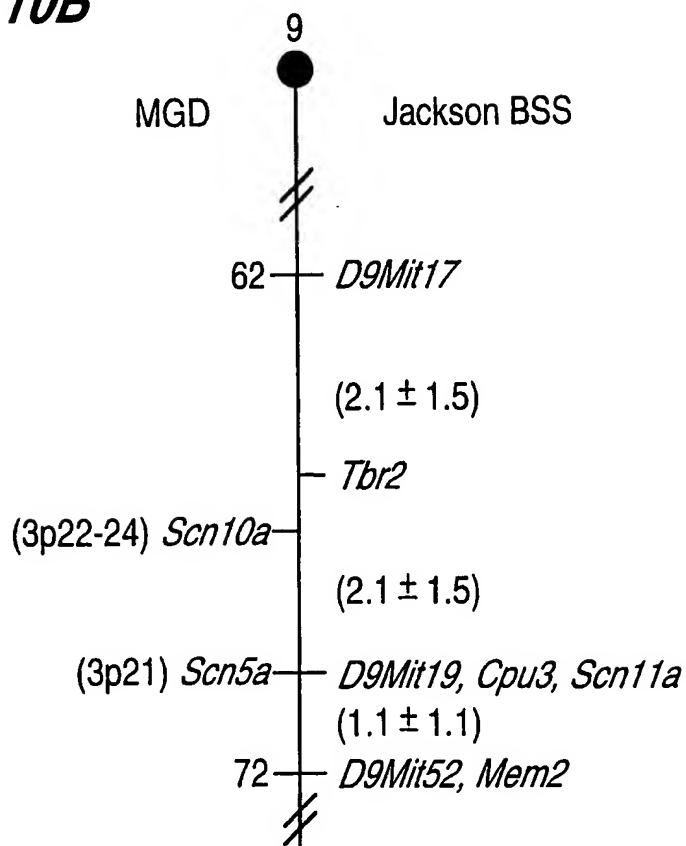
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# ***FIG. 9***

NaN immunostaining in DRG neurons



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**FIG. 10A****FIG. 10B**

# PATENT COOPERATION TREATY

## PCT

### DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT (PCT Article 17(2)(a) and Rule 39)

Applicant's or agent's file reference 44574-5004WO	<b>IMPORTANT DECLARATION</b>	Date of mailing (day/month/year)
International application No. PCT/US99/02008	International filing date (day/month/year) 29 JANUARY 1999	(Earliest) Priority Date (day/month/year) 29 JANUARY 1998
International Patent Classification (IPC) or both national classification and IPC C07K 14/435; C12N 5/16, 15/12, 15/63; G01N 33/53		
Applicant YALE UNIVERSITY		

This International Searching Authority hereby declares, according to Article 17(2)(a), that no international search report will be established on the international application for the reasons indicated below.

1. ☐ The subject matter of the international application relates to:
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  - b. ☐ mathematical theories.
  - c. ☐ plant varieties.
  - d. ☐ animal varieties.
  - e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes.
  - f. ☐ schemes, rules or methods of doing business.
  - g. ☐ schemes, rules or methods of performing purely mental acts.
  - h. ☐ schemes, rules or methods of playing games.
  - i. ☐ methods for treatment of the human body by surgery or therapy.
  - j. ☐ methods for treatment of the animal body by surgery or therapy.
  - k. ☐ diagnostic methods practiced on the human or animal body.
  - l. ☐ mere presentations of information.
  - m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art.
2. ☐ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:
 

☐ the description
 ☐ the claims
 ☐ the drawings
3. ☒ The failure of the nucleotide and/or amino acid sequence listing to comply with the prescribed requirements prevents a meaningful search from being carried out:
 

☒ it does not comply with the prescribed standard  
☒ it is not in the prescribed machine readable form
4. Further comments:  
Please See Continuation Sheet.

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**DECLARATION OF NON-ESTABLISHMENT OF  
INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US99/02008

**4. Further Comments (Continued):**

The computer readable form for this case is defective. Claims refer to sequences for which a computer readable form of the sequences are required for a search. Claims cast with a reference to the structure of a "NaN" cannot be searched because the specification defines NaN in terms which depend on the sequence. The search of the special technical feature of the invention requires the computer readable form of the sequence.

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